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BIOLOGICAL BULLETIN

THE EFFECT OF X-RAYS ON THE RATE OF CELL DIVISION IN THE EARLY CLEAVAGE OF PLANORBIS.[1]

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Only a very short time had elapsed after the announcements of the discovery of X-rays (in 1895) and of radium (in 1898), when it became generally known that animal and plant life can be profoundly affected by radioactivity. Subsequently, a large amount of experimentation has been done and many interesting results prove the powerful action of these agents upon living matter. Physiological and therapeutic studies of radioactivity have long since given a firm empirical foundation for its application in the cure of disease. From the pure biological standpoint, also, experimentation has not been lacking; instead, a long list of titles stands to its credit.

However, most of this purely biological work has concerned itself with the production of abnormalities either in the embryo or in the adult. Only recently has there been any attempt to analyze these results from the study of the units which make up the tissues affected. Nevertheless, it seems clear that the effects upon an organism of radioactivity, or of any agent which produces abnormalities, must depend very largely for any real explanation on results obtained from the study of the effects of that agent upon the cellular elements making up the organism. In the present cases the character of the animals studied accounts for the lack of data at hand upon the more detailed effects on the cells of the tissues in question. Generally vertebrates have been chosen as subjects of experiment and observation and their cells

[1] Contribution from the Zoological Laboratory of the University of Texas, No 117.

with a few exceptions have not proven suitable for a study of cytological details.

With the idea of finding more exactly what occurs to individual cells when exposed to X-rays, the writer studied the eggs of the freshwater snail, *Planorbis lentus*, in relation to this problem. By choosing such a form, several advantages are gained. The eggs divide in a very definite manner and the normal course of their development has been carefully observed, as has that of many related gasteropods. It is, therefore, a comparatively simple matter to study at least the more gross effects of the exposures, and to compare with experiments of most varied character upon forms not dissimilar in the details of their development to the one here employed.

Furthermore, there is hope that the use of radioactivity in experiments on eggs of well-known type may lead to further knowledge of the principles of egg structure and organization. The reactions which the eggs give to exposure to X-rays must be, if constant, the expression of some quite definite mechanism within the egg to which the X-rays act as a stimulus. A comparison of these reactions with the responses of this mechanism to other stimuli of different nature very possibly may lead to interesting conclusions as to the nature of the mechanism itself. This, of course, is the much broader biological problem.

In most of the work which has been done recently from the standpoint of pure biology radium has been the agent used for experimentation. In general one would expect that the results obtained from radium rays would be similar to those from X-rays; but it is not possible to predict that such is the case and the results with radium have been comparatively meager. Radium rays are of three kinds, α, β, and γ; of these the γ rays are the more penetrating and to them are probably due most of the effects on living forms. From comparative studies made by physicists it is well known that the γ rays of radium are quite similar in many particulars to the X-rays, and it is stated by Rutherford that they are in fact the more penetrating X-rays. In view of the facts, therefore, that it is perhaps easier to understand something of the nature of the disturbances caused by the X-rays, and that this form of radio-activity is more easily

obtainable at this laboratory, it was determined to use X-rays rather than radium. No difficulty has been experienced in getting results with the X-rays.

The work which is here reported was carried on at the University of Texas and at the laboratory of the U. S. Bureau of Fisheries at Woods Hole, Mass. The Bureau of Fisheries has kindly given permission for the publication of results obtained at its laboratory.

For the use of the X-ray machine, which he kindly loaned me, and for his assistance in various ways during the early course of these experiments, I am indebted to Mr. Oliver Brush, of Austin, Texas.

The snails used were identified for me as *Planorbis lentus* Say by Dr. W. H. Dall, of the Smithsonian Institution, to whom I wish to express my obligation. They were secured from Waller Creek, a small stream near the University of Texas.

These experiments were conducted during the early part of the year 1913. After the results had been studied and written up, it appeared wise to delay publication until another breeding season could furnish new material and further experiments could be carried on in order to extend the observations and perhaps give rise to broader conclusions. During December, 1913, however, Texas was visited by one of the most severe floods in its history and the streams were cleanly scoured out. Conditions of vegetation also were greatly changed. As a result, where formerly *Planorbis* had been found most numerously during the spring months, there are now only a very few scattered specimens. Furthermore, much difficulty has been experienced in getting these specimens to produce eggs. For these reasons I have been unable to renew the experiments on *Planorbis*. Other fresh water snails suffered largely the same fate during the flood, but their pointed shape enabled more of them to maintain themselves against it, and I have been able, therefore, to study somewhat the effects of the rays on *Physa halei*. As these eggs are less suitable for detailed study in the living condition owing to the thickness of their gelatinous covering, the statements made in this account apply chiefly to the eggs of *Planorbis*. In general, the behavior under exposure of the eggs of these two species has not been

found to differ much. Reference to these more recent observations will be made in the appropriate places under the later discussion.

METHODS.

Specimens of *Planorbis* may be kept in aquaria, and during the night they will lay on the sides of the glass dishes or on the water plants that may be in the aquaria. If lily pads are placed in the aquaria, their rough lower surfaces seem to be favorable places for finding clusters of these eggs. The eggs occur in "clusters" (Holmes) which are bound together by tough enclosing membranes and which contain a considerable amount of jelly. Within the clusters there are, perhaps, a couple of dozen capsules filled with yellow albumen mass in which the *Planorbis* egg itself develops.[1]

The eggs of *Planorbis* are mostly laid at night, or usually just before day. Observations made early in the morning, say 7

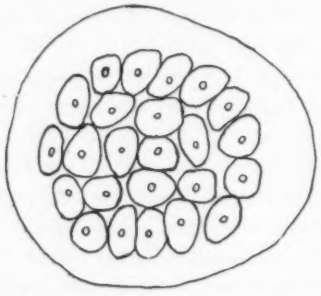

FIG. 1. Egg cluster of *Planorbis*, showing eggs within the albumen capsules, all of which are surrounded by a gelatinous mass. (From Holmes.)

A.M., frequently find eggs which have not yet put out the first polar body.

In studying the eggs of *Planorbis*, the general method of procedure has been as follows: the clusters of eggs have been removed from the sides of the glass dish or from the plants upon which they were deposited by inserting a sharp knife between them and the attachment, care being taken not to break the gelatinous capsules. The eggs can now be studied under conditions which do not differ

[1] See Holmes's description, *Jour. Morph.*, Vol. 16.

widely from the normal by merely placing them in a watch glass, and observing them under a microscope. To expose the eggs to X-rays it is only necessary to set the watch glass under the X-ray tube.

When considering the results of these experiments and the effects obtained on *Planorbis* eggs it must be kept in mind that these eggs are normally subjected to wide variation in temperature—from mild winter temperature to summer heat—and they are unusually well protected. The description given by Holmes (p. 375) of the unsegmented eggs of *Planorbis trivolvis* fits the egg of *Planorbis lentus* accurately. He describes the egg as embedded in a thick albumen mass within a capsule, which in turn is surrounded by a jelly mass, and the whole including a score of other similar capsules, is covered by a tough enclosing membrane. In making the experiment the entire cluster only is disturbed. Thus there are eliminated from consideration such factors as changes of temperature, of pressure, of oxygen or carbon dioxide content, etc. In fact the eggs were developing under entirely normal conditions, with the exception that the cluster had been freed from its attachment, at the time of exposure. The only factors that this loosening and the subsequent treatment of the eggs could involve, except the factor under experiment, would be a very slight degree of shaking, changes in the direction of the attraction of gravitation, and changes in light intensity; and it is highly improbable that either of these would have much effect upon the rate of development, or upon the finer structure of the egg.

When it is desired to fix the eggs it is only necessary to break the capsule with a needle and tease them out. I have teased them as Holmes recommends into salt solution which contained a little picric acid and then fixed them in Kleinenberg's picro-sulphuric.

Under proper conditions of illumination, it is usually possible to observe the grosser details of spindle and aster formation in the living egg, and the early cleavages are not difficult to follow. The normal course of cleavage is approximately as described by Holmes ('00) for *Planorbis trivolvis* and by Conklin for *Crepidula* ('97). The cytological details as worked out on the

latter animal (Conklin, '02) apply to a large extent to the development of *Planorbis*.

The progress of the division as seen in the living egg is briefly as follows. The nucleus can be seen in the resting state as a clear spherical area distinguishable from the rest of the cytoplasm by its lighter color. As the spindle progresses the egg elongates slightly in the axis of the spindle in preparation for division. In the succeeding cleavage this elongation is more clearly marked.

After the furrow has separated the blastomeres, they round up until they are almost spherical in form and the contact surface forms only a narrow protoplasmic bridge between them. This rounding off of the blastomeres is repeated after each cleavage, at least after the earlier cleavages, and suggests, as Holmes points out, the result of some tension which is exercised at the poles of the eggs. The tension soon appears to decrease, however, for the blastomeres flatten against each other gradually as if they were being drawn together, with the result that each assumes a more or less spherical form and the contact surface becomes a mere line between the eggs. This flattening is accomplished by a rotation of the blastomeres toward the animal pole. The nuclei and the asters thus come to lie near the animal pole and the spindle is bent sharply (cf. Conklin, '02). Presently, after the blastomeres have flattened against each other in the two-cell stage, a "lenticular cleavage cavity" (Holmes) appears between them, and its maximum is a criterion as to the progress of the next stage.

Since these stages occur with little variation and can be seen rather clearly in the living condition, it is possible to expose the egg to the X-rays at almost any time desired.

During the resting stage the nuclei when viewed from the animal pole lie very near the furrow. Thus the two nuclei of the separate blastomeres are brought as close to each other as possible. At a little later stage the nucleus in each blastomere passes down toward the center.

The cytoplasmic constriction cuts in deeper at the animal pole on the first cleavage as Holmes has pointed out; this is the general rule with eggs of this type. It holds true for the suc-

ceeding cleavages, but in cases of some eggs which have been exposed to the X-rays I have noticed that the second furrow is the one to cut in more deeply.

When the nuclei of the blastomeres have moved to the center they begin the second division. By the time the cytoplasmic division can be seen the nuclear division is well under way. It is the rule to which exceptions are only occasionally seen that the two cells should divide at the same time. However, it is true that one cell will sometimes divide before the other has progressed far, and a three-cell condition results as shown in Plate I., Fig. 8, of Conklin's ('97) paper.

The blastomeres again elongate in the direction of the long axis of the spindle. The furrow begins to cut in from the sides and from the animal poles. The cells again round off after the division, and we again find the cleavage cavity present. It differs from the first cleavage cavity, however; in addition to the lenticular spaces which appear between each pair of dividing cells, a large rectangular cavity is to be seen at the center.

The third cleavage consists in the giving off of the first quartette of micromeres (ectomeres) at the animal pole. A spiral dextrotropic shifting of the quartette occurs after which the cells flatten; and there remains once more an almost spherical mass of cells. A central cleavage cavity again makes its appearance and again disappears after the next cleavage.

The four macromeres divide now to produce a second quartette of micromeres; thus arises the twelve-cell stage. As before, after the division the cells flatten to form a spherical mass. In the living condition it is most easy to determine which stage is being observed when one can see the egg in an optical section running from pole to pole. The division of the first quartette of ectomeres now produces the sixteen-cell stage which in turn give rise to the twenty-four-cell stage by division of the second quartette of ectomeres and at the same time a division of the macromeres by which the third quartette of ectomeres is given off. According to Holmes "the twenty-four-cell stage, which is reached by these divisions, marks a resting stage of considerable length in the development of the egg. A cleavage cavity is formed at this time which may acquire quite a large size" (page 380). This is the

stage at which in general observations on the living eggs as to the effect of exposure to the X-rays was closed.

To sum up: the first division produces the cells *AB*, and *CD;* the second division *A*, *B*, *C*, and *D;* the third division 1*A*, 1*a;* the next, which is the 12-cell stage, consists of 2*A*, 1*a*, 2*a;* the 16-cell stage consists of 2*A*, 1a^1, 1a^2, 2*a;* the 24-cell stage consists of 3*A*, 1a^1, 1a^2, 2a^1, 2a^2, and 3*a*.

Experiments and Observations.

Eggs of *Planorbis* were exposed to the X-rays while immersed in water in a watch glass. First they were carefully observed under a microscope and the degree of their development noted. The glass containing them was then placed at a distance of about four inches below a tube of average hardness (after the current had been started through the tube). An automatic regulating device on the tube used made possible a fairly uniform exposure. At the close of the exposure the eggs were again examined and the degree of development noted. Observations were then made at intervals during the day as convenience permitted. Usually at the end of five or six hours the eggs had reached a stage where further observation of them in the living condition was unprofitable. At this stage they were usually killed and fixed, although some were allowed to develop for later study.

In the cell divisions which are concerned in the maturation and cleavage of *Planorbis* eggs up to the twenty-four cell stage the writer has never observed any division under usual conditions of temperature to take place in less than forty-five minutes. It is, moreover, exceptional to find the division occurring in so short a time as this, for in general the complete cycle does not take place under an hour and often it is longer than that. Exact data for a table showing just how long a period of time elapses between each cleavage is not at hand,[1] but my records show no

[1] The following figures are taken from an observation made under average conditions. They may be regarded as normal, but there is variation from this norm as stated above.

Experiment (1) The 1st cleavage division required 1½ hours for completion.
2d cleavage division required 1¼ hours for completion.
3d cleavage division required 1¼ hours for completion.
4th cleavage division required 1¼ hours for completion.
5th cleavage division required about 1 hour for completion.

case in which the division was completed in less than forty-five minutes. On the other hand, cases have been observed in which fully two hours elapsed before a second cycle began.

It is, of course, true that the rate of division can be changed by varying temperature. Eggs which are kept in a refrigerator will require several hours to complete a division. Similarly, eggs will divide much more rapidly upon a warm day. The statements made in the preceding paragraph, however, are based upon conditions which obtain normally, or at least upon conditions which are as near normal as it is possible to come in the laboratory.

It may be conservatively stated that the range for these early cleavages is from fifty minutes to more than two hours.

If *Planorbis* eggs are exposed to the rays during the resting stage between two mitoses the results are less marked than if the exposure is during the progress of the mitosis.[1] It is quite certain from the later behavior of eggs so treated that no ex-

Incomplete observations on *Physa* eggs seem to indicate a similar range of time values for the cleavage divisions in that form.

(This observation and those included in the succeeding footnotes form only a part of the data taken in this investigation. The arrangement here and the number of the various experiments indicate nothing more than convenience for reference. The experiments, of which there were many more than are here given, were not made in this order; these are chosen merely as examples bearing on the points under discussion.)

[1] (2) At the beginning of this exposure, the eggs had just finished the first cleavage and their nuclei were resting. They were exposed to the X-rays for six minutes. At the end of this time there were no visible effects. Thirty-five minutes later the second furrow made its appearance and in one hour and fifteen minutes after the exposure, the second division was complete. Two and one-half hours were required for the next division, a much longer time than normal.

(3) The eggs used in this experiment had been observed during the progress of the first cleavage division, and were exposed for six minutes at the time when the blastomeres were most widely separated and the nuclei were resting. The exposure had no visible effect, and the eggs apparently did not depart from their normal course. One hour and fifty minutes, however, were required for the next cleavage. The third cleavage consumed one hour and fifteen minutes, and in two hours more the sixteen cell state had been reached.

(4) At the time of exposure the first cleavage furrow had separated the blastomeres fully. The exposure lasted three minutes and produced no effect except that ten minutes after it had begun the blastomeres had flattened against each other. One hour and ten minutes after the exposure, the second furrow made its appearance in some of the eggs. Not all had progressed equally, some having nearly completed the second division. The first quartette had been given off and its division begun, at the end of one hour and forty five minutes more.

Other cases might be cited.

posure is without some effect, but whatever changes there may be induced are very slight if the exposure is during a resting stage. Only a slight stimulation of rather uncertain nature can be produced. It does not manifest itself by changing appreciably the rate of division so far as hastening is concerned; the evidence, however, would indicate that later on the phase of depression (see below) follows such an exposure although it may be in less degree. Cytological examination shows only slight effects of exposure during the resting stage on the structure of the protoplasm.

It is probably not misleading to say that during the resting stage the egg is in a state approaching equilibrium in which activation is with difficulty produced.

Recent experiments on developing eggs have in general given results similar to those just outlined. Conklin ('13) found abundant proof in his *Crepidula* experiments of the principle enunciated some years ago that dividing nuclei are more easily disturbed by environmental change than nuclei at rest. Koernicke ('05) noted that after an exposure of the roots of *Vicia Faba* and of *Pisum sativum* for two days to radium the resting nuclei appeared unaffected. This general result has been obtained so widely that it seems unnecessary to cite further proof for a position against which there is no contradictory evidence.

Following the general rule that resting nuclei are only with difficulty stimulated one would expect little result from stimulating eggs in the germinal vesicle stage. As far as effect on the rate of cell division is concerned exactly that result was obtained.[1] Fertilization had of course already occurred, for the sperm enters *Planorbis* eggs at the time of laying. The egg, therefore, at the time of exposure was beginning a new cycle of development, caused by the entrance of the sperm, but its nucleus had not yet started upon its cycle and so was not disturbed by the stimulation.

[1] (5) The eggs in this experiment were in the germinal vesicle stage at the time of the exposure, which lasted 20 seconds. At the end of the exposure no change could be noted. One hour and thirty minutes later the maturation divisions had passed and the first cleavage furrow was making its appearance. One hour later the second cleavage was nearing completion. Four and one half hours later the first quartette had divided.

(6) Eggs in germinal vesicle stage were exposed three minutes with no visible effect. Four and one half hours later they were killed and fixed in the four cell stage.

Similarly at the end of the maturation divisions before the fusion of the male and female pronuclei no marked stimulation could be produced by exposure to the X-rays.

Contrasting with the condition just described is that found in the cleavage mitosis. If one may speak of the cell in the resting nucleus stage as being in a state of equilibrium or stability with respect to its capacity to respond to stimulation, we may likewise say that during a mitosis it is relatively unstable with respect to this particular character;[1] and there appears to be a certain time during the course of spindle formation when the capacity of the cell to respond is greatest. During the period from the definite formation of the spindle to the metaphase or anaphase, response to X-ray stimulation is easiest to obtain. Similar results have been reported for experiments with various means of stimulating the egg. Conklin ('13) found much clear evidence to prove that "the early stages of cleavage are more sensitive to environmental changes than later ones."

[1] (7) The eggs of this experiment had just completed the formation of the first polar body and the second maturation spindle was beginning to form when they were exposed to X-rays for ten minutes. At the end of the exposure the second division seemed to be entirely complete. The first cleavage division was accomplished in the next fifty minutes. The succeeding divisions occupied more time, for one hour and thirty minutes elapsed before the completion of the second cleavage, and a like period of time passed before there was any sign of a micromere division. Later divisions were even more slow. The control was far ahead in its divisions, having reached a stage where the blastomeres could no longer be counted accurately in the living condition.

(8) The eggs of this experiment were exposed ten minutes during early stages of the second cleavage spindle. When the exposure was ended it was found that in many cases the division was completed, and in all it was well along. The third division occurred in thirty five minutes more. Owing to an accident, further data on this set of eggs were not obtainable.

(9) The eggs in this set were secured during the spindle formation of the first maturation division. The exposure lasted six minutes; at its conclusion, the cytoplasm could be seen collected largely at one pole of the egg, and in some cases a polar body elevation was apparently beginning to form. In half an hour both maturation divisions were completed. One hour and twenty minutes were consumed in the first cleavage division and one hour and fifty minutes more in the second division. Two hours later no advance was noted.

(10) Eggs in the late prophase of the first cleavage were exposed three minutes, at the end of which time they were found to have completed the division, and in five minutes more the blastomeres had flattened against each other with the peculiar lenticular cavity between them. In thirty minutes the second cleavage had occured and in two hours and a half the fourth had been accomplished.

Variations in the results of exposing the eggs are probably to be explained largely on the basis of this maximum and minimum capacity for response.[1] In other words, if the exposure is made during a resting stage, the minimum stimulation results; if it is made during the period of the early spindle, the maximum stimulation is obtained; but if it is made between these two extremes, the result is neither maximal nor minimal and there is only a partial degree of stimulation, the amount depending upon the relative position of the nuclei in the mitotic cycle when the exposure took place.

The first visible effect on *Planorbis* eggs of exposure to X-rays is to stimulate their division. Any particular mitosis which may be in progress at the time of the division is hastened to a very great degree.[2] So far as may be observed in the living egg the process is not different in character from the normal indirect cell division—although the later events in the life of the egg make it certain that something essential in the mechanism has been disturbed—but the time which is consumed by a cell

[1] (11) At the time of the exposure of three minutes these eggs showed no sign of division, nor was any change noted at the end of the exposure, although in some cases, what were presumable early maturation spindles, could be seen. One hour and ten minutes later the first cleavage division had taken place, and in forty five minutes more the second furrow had appeared. In one hour from this time the first micromere division had not been completed, but in two and one quarter hours after the second furrow the second micromere quartette had been given off, making the twelve-cell stage.

Experiments (12) and (13) were exact duplicates of (11).

[2] Compare experiments (8), (10).

(14) The eggs of this set showed the first trace of the second cleavage furrow at the time of the exposure, which lasted ten minutes. At the end, the eggs were all in the four cell stage. While not all of the cluster had been exactly together at the beginning of the exposure, all apparently finished together.

(15) At the end of a six minute exposure, eggs in which the second furrow was barely visible at the beginning had now passed into the resting condition. In thirty minutes more the first micromere quartette had been completely given off. Two hours later the eggs were probably in the 16-cell stage, but it was not possible to observe exactly in this case.

(16) Eggs in the 4-cell stage were exposed three minutes. At the end of the exposure the first micromere quartette had been given off, although in a few cases not quite completely. The four macromeres were as yet spherical, not having flattened against each other; thus they gave the appearance of being almost completely separated from each other. Twenty five minutes later the second quartette had been given off, and at the end of another half hour the first had divided. One hour and thirty minutes later the egg had reached a stage comparable to Holmes' 24-cell stage, for which see Fig. 10.

undergoing mitosis at the time of the exposure is very much less than in the case of a cell dividing under more normal conditions. Thus, of the effects induced in the egg by exposure to X-rays, the first takes the form of a marked increase in the activity of the egg, causing a phase of acceleration. This effect was first obtained after eggs had been exposed ten minutes, when it was noticed that divisions had actually been completed in cells where only a spindle was to be seen at the time the exposure began. That is, during an exposure of ten minutes there had been accomplished a complete process which never under normal conditions had been observed in this form to occur in much less than an hour. I have repeated this observation from January to June on many experiments and have obtained the result without variation. Whenever an egg of *Planorbis* in any cleavage up to the sixth, farther than which it is not practical to carry on observations on the living egg, is exposed to X-rays any mitosis which may have been started is hastened to its completion, and in almost every case that state has been reached by the time the egg can be taken from under the tube and examined under the microscope.

Subsequently, I have reduced the length of the stimulation to six minutes, five minutes, and three minutes without noticeable difference in the result. In each case the mitosis (both nuclear and cytoplasmic divisions) in question was nearly if not quite completed at the end of the exposure. Even shorter exposure than this will bring about the result more or less completely. I have exposed the eggs as short a time as twenty seconds and have found the phase of acceleration almost as marked although the cell division would not be fully completed at the end of the exposure. Thus it is seen that a very short exposure only is necessary to induce the acceleration.[1] Comparing this induced result

[1] Compare experiment No. 16. (17) At the time of exposure the eggs of this experiment were in the early stages of the second cleavage division, but were not at all in the same stage of advancement. (Some had not quite completed the first division.) The exposure lasted 20 seconds, and at the end of it the eggs were examined as quickly as possible. Many had almost completed the second cleavage and others had passed well into it. Only a few, which were probably in the resting condition at the time of the exposure, showed no effects. Fifteen minutes later nearly all had completed the second division and their nuclei were resting. Forty five minutes later the beginning of the first micromere divisions were visible but

with the normal, it appears that only a small fraction of the time usually taken is consumed under the conditions of the experiment.

It has been difficult so far to get much information concerning the phase of acceleration. It is passed very quickly; in the last cases cited scarcely more than a minute was consumed, and in no instance was more than ten minutes of exposure necessary to bring about the result. It seems that there is a minimum time in which a cell division in the early cleavages of the egg of *Planorbis* can take place—actually something more than a minute—and that the stimulation by exposure to the rays need be but very short in order to reduce the time from normal to this minimum.

Aside from their bearing on the effect of X-rays on the living organism, the facts connected with the shortening of the time necessary for mitosis have considerable interest from their bearing on the questions of cell mechanism.

The first effect, then, of exposure on the rate of cleavage is to stimulate greatly whatever mitosis may be in progress and to hurry the cells into the resting stage.

The effect of exposing a cluster of eggs not all equally advanced forms an interesting corollary to the observations on the induced acceleration of individual eggs. The usual conditions in *Planorbis* as in most forms, is that all the eggs in a cluster are in the same stages of development. However, there sometimes occurs a variation from the general condition and of the two dozen eggs

thirty minutes yet were consumed before the divisions were completed. An hour and a half later the eggs were in the 12-cell stage.

(18) Eggs in the four cell stage were exposed twenty seconds. Five minutes elapsed before they were examined but at the end of that time the first micromere quartette had been nearly, if not completely, divided off. An hour later the second quartette had appeared and in another thirty minutes the first quartette had divided. Seven minutes later a second exposure of twenty seconds was made, at the end of which in at least part of the eggs the second quartette had divided. It was difficult to see cell boundaries after this stage, but the third quartette could not clearly be seen until after another hour and a quarter had elapsed.

(19) The eggs of the cluster were well along in the second division at the time of exposure, which lasted twenty seconds. When they were examined (as soon as possible after the exposure) the division seemed to be entirely finished. Thirty five minutes later the first quartette was given off, and was followed by the second after another thirty five minutes. A second exposure of twenty seconds was now given; at the end of it, in part of the cells at least, the first quartette had divided. An hour and a quarter elapsed before the end of the next division.

present some one may often be found in nearly every stage of the cleavage division in progress at that time. Reference is here made particularly to the first or second cleavage. But it is very unusual to find eggs in the cluster in two clearly distinct cleavages at once. If a cluster with various stages represented is exposed to the rays, the effect as observed at the end of the exposure is to equalize the progress by hastening all of the eggs, except any which might not have begun the division at all, to the completion of the mitosis and into the resting stage.[1] That is, if the exposure found some of the eggs of a cluster in an early stage of mitosis and others in a later, it would, by inducing the acceleration in each individual bring practically all into the resting stage at the close of the radiation.

It cannot be affirmed, however, from my observations that subsequent divisions of such a cluster as that just described would of necessity occur exactly at the same time.

This observation on a living egg that the divisions are greatly stimulated by the X-rays goes to explain the observed fact that in fixed eggs which have been exposed, mitoses are not so easy to find as in eggs which have not been exposed.

The phase of acceleration does not last long in these cells but passes off at the end of the first division or perhaps the second after the exposure. Following it there sets in without further exposure a phase of depression, during which the rate of cell division becomes slower and slower.[2] The eggs' activity as regards cell division is markedly inhibited. This invariably occurs, although the extent and nature of the inhibition or depression may not be exactly the same in all cases. This depression may amount to a complete stopping of cell division, thus terminating the experiment; or often observation has been interrupted that the eggs might be fixed for cytological examination.

The depression phase occurs without regard to the stage of the development of the egg at which the exposure took place and,

[1] Compare experiment No. 14. (20) The eggs of the cluster were going through the first cleavage division but had not all progressed equally in the division. The exposure lasted twenty seconds, and the eggs had nearly all completed the division at the end of it. Forty minutes later the blastomeres had flattened against each other and an early spindle was to be seen.

[2] Compare experiments (2), (7), and (15).

so far as now determined, without regard to which particular cleavage is in progress. The whole question of the effect of the rays so far as the rate of division is concerned is not one of the state of progress of the egg in cleavage but one of the mitotic cycle. As long as the exposure is made at the same relative time in the mitosis it makes no difference with which of the early cleavage divisions the experiment begins. It is as though the energy of the eggs was used up upon exposure to the rays in hurrying the eggs into the resting condition and that continuously more and more time is required to raise the cell to the point where it can again divide. Or perhaps it may be plausible to explain the result on the grounds that the X-rays have a double effect: first a stimulative effect, and second, a very injurious inhibitive effect. The former effect is produced during what may be called the latent period of the latter. That there is some particular factor concerned more than mere stimulation is seen from the experiment described below in which a second phase of acceleration and the second phase of depression was obtained by a second exposure. The second stimulation was less and the depression more rapid than the first. Now, if the stimulation were all that had taken place, as for instance in a muscle-lever experiment where the results of *simple* stimulation are obtained, the second stimulus should have been quite as effective as the first; such however was not the case.

It has already been stated that very little effect results from exposing the eggs in the resting stage; however, there is some evidence for thinking that such an exposure causes a depression in the rate of division after it. The data at hand on this point are not as positive as desirable, but it seems to indicate that conclusion. In two or three hours after the exposure the divisions became slower and slower as they would have done (but to a much less degree) if the egg had been exposed during mitosis.

There is one case forming an apparent exception to the general observations as stated above. If the exposure occurs during the first maturation division, the depression does not set in until the first cleavage mitosis.[1] The first and second maturation divisions take place with considerable rapidity and show the effects of

[1] Compare experiment (9).

the stimulation. This, however, is the condition one would expect in view of the fact that a complete mitotic cycle has not elapsed since the exposure, for there is of course no resting vesicular nucleus stage between the maturation divisions. It is, therefore, not a real exception, but on the contrary is quite in line with the other observations.

Eggs which have been exposed to X-rays and have passed into the depression phase may again be stimulated by a new exposure to the rays.[1] These exposures may both be as short as twenty seconds and they are both subject to the conditions previously described, but there is no question as to whether the effect will be produced. However, the new phase of acceleration is not so great nor so clearly marked as the first, while the phase of depression comes on sooner and takes place more rapidly than in the case of the first exposure.

The relation which the phase of depression bears to the normal development may be illustrated graphically. The following data, plotted in Fig. 2, are from a representative experiment.

	Control.	Experiment.	Experiment is Faster than Control.
1st div.....	75 min.	3 min.	+72 min.
2d div.....	55 min.	32 min.	+23 min.
3d div.....	80 min.	60 min.	+20 min.
4th div.....	70 min.	90 min.	Slower −20 min.
5th div.....	65 min.	100 min.	Slower −35 min.

The control or normal is used as a base line and the variations of the exposed eggs from the control are plotted with respect to it. The curve, of course, does not show the phase of acceleration. It indicates what has already been set forth, that owing to the depression the time required for a division gradually lengthens relatively until it is equal to the normal rate, and then falls below. The divisions get relatively slower and slower.

The phase of depression is of sufficient interest to warrant more extended study. To what extent it occurs and how far it may be carried with recovery of the eggs are questions to which I cannot now give a satisfactory answer.

[1] Compare experiments (18) and (19).

A control,[1] whether part of the same cluster or another cluster in the same stage of development, may be started at the time the exposure is made. If the control is observed after an interval of several hours, it is found ahead of the radiated eggs in development. If, however, the control as well as the radiated eggs be observed at more frequent intervals, quite a different state of affairs is to be seen. During the first two or three divisions after the exposure the radiated eggs are ahead of the control. They gradually get slower, however, as already explained, while control maintains its normal course. Not only do the radiated

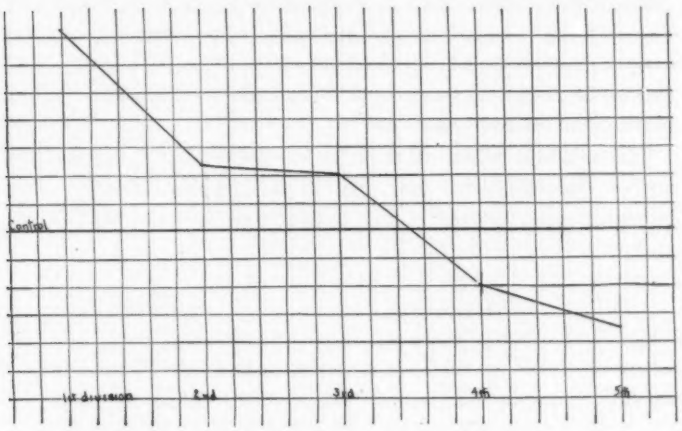

FIG. 2.

eggs slow down to the normal rate so that for a very short time both they and the control progress together, but presently they become even slower. Usually by the time the twenty-four cell stage is reached, if the exposure was during the first or second cleavage, the control has more than passed the radiated eggs in the degree of its development. This is explainable on the basis of the observations previously noted. First the divisions of the eggs are stimulated by the exposure, during which time they get ahead of the normal eggs; then the depression phase sets in and

[1] Compare experiments (1) and (7). References to experiments in point might be multiplied greatly, but those given are thought to be sufficient to illustrate the principles set forth.

they gradually get slower and slower until they are developing less rapidly than the normal eggs. Thus the last result is a retardation of growth and cell division.

The following experiment on the eggs of *Physa* gives additional evidence in support of the conclusions just stated and also suggests certain other effects of the rays. There are three sets of data given, and the eggs used were all from the same cluster. All were in the same stage at the beginning of the experiment. In the first column is shown the rate of development of the control; in the second, are the stages for corresponding times of the eggs which were exposed five minutes to the X-rays; and in the third are similar observations for eggs exposed ten minutes. It will be seen that in the shorter exposure the divisions are going more rapidly than the control, but at the end of the experiment they

Time Intervals.	Control Eggs.	Eggs Exposed to X-rays 5 Min.	Eggs Exposed to X-rays 10 Min.
Beginning of experiment.	All eggs in the early stages of the first cleavage.		
		At the end of the exposure the first cleavage furrow was cutting in.	
+6 min.	1st cleavage furrow not yet visible.	1st cleavage completed.	At end of exposure there was no external sign of division.
+18 min.	1st cleavage furrow beginning to divide the eggs.	Blastomeres flattened against each other.	One egg divided, others show no external signs.
+20 min.	1st division completed.	No record.	Furrow beginning to cut in.
+15 min.	Blastomeres flattening against each other.	4-cell stage nearly completed.	2-cell stage nearly completed.
+130 min.	8-cell stage.	12-cell stage.	4-cell stage.
+4½ hrs.	16–20 cell stage.	24-cell stage.	12-cell stage.

were getting slower. These radiations were made with an apparatus carrying a stronger current and giving off more intense rays than hithertofore. To that condition is without doubt due the effects produced by the stronger radiation. In this last case

the depression set in before the acceleration could take place and during the entire course of the experiment these eggs were behind both the shorter radiation and the control in development.

As is well known temperature profoundly affects the rate of cleavage, a rise causing an increase in the rate. The question at once is suggested, can the temperature changes induced by the conditions of the experiment account for the effects on the rate as here given. There are several considerations which would seem to point to an affirmative answer to this question. (1) The resistance which the rays meet in passing through the protoplasm of the egg would tend to cause a rise in temperature. (2) It is easily conceivable that some of the energy might be converted into heat energy. Rutherford has shown that radium emanination produces a rise in temperature of considerable extent in gases through which it passes. (This is due, however, to the α rays to the extent of 70 per cent. of the effect noticed). (3) Finally, the histological conditions in the eggs resemble those produced by allowing eggs of *Crepidula* to develop at a temperature six degrees higher than normal (see Conklin, '12). However, in regard to the last point, it is to be remembered that in modifying the development of eggs a given result can often be produced by several different means.

There are on the other hand considerations which make it impossible to account for the effects on the basis of temperature changes. *Planorbis* lays its eggs in shallow water from late winter up into the early summer, and the eggs are adapted to undergo wide changes in temperature without ill effects. They are very small and when the exposures were made were well covered with water in addition to the insulation afforded them by their gelatine and albumen coverings, and furthermore the exposures were of short duration. It is difficult to see, therefore, how sufficient rise in temperature to bring about the marked results herein described could be produced.

Finally, and this seems to be the test experiment, it cannot be that heating causes the effect, because the X-rays produce the acceleration whether the exposure is longer or shorter. There seems to be no marked difference in the effects of a ten minute exposure and one of three minutes, while one of but twenty

seconds is almost as effective. Obviously, if rise in temperature were the cause of the more rapid division, a long exposure would give a more rapid cleavage than a very brief radiation.

Recently the writer has been carrying on some experiments, the results of which will be published in another place, to ascertain the effect of X-rays upon certain enzymes. The general conclusion drawn from these experiments is that the activity of the enzymes in question is increased somewhat by a weak exposure, but decreased by a stronger radiation. In the light of the study on cell division here reported and especially of such observations as those of the effects of the stronger radiation on *Physa* eggs, the suggestion of a possible relation between these two sets of phenomena forces itself upon one. In a late paper, Packard suggests "that radium radiations act indirectly on the chromatin and the protoplasm by activating enzymes." This is not unlikely the case, in the writer's opinion, and is in harmony with the observations here presented, for the effects of radium rays appear to be comparable only to those of weak X-rays. It is of course by no means clear how the activation of the enzymes takes place.

Summary of the Observations on Experiments.

1. The eggs of *Planorbis* require normally from fifty-five minutes to about two hours to complete a division (up to the twenty-four cell stage). In no case has a division been observed to occur in less than forty-five minutes.

2. By exposure to X-rays during the resting stage of the nucleus only a very slight stimulation may be produced.

3. Exposure during the early part of the formation of the mitotic spindle is most effective.

4. The first effect of exposure upon the rate of cleavage is to stimulate mitotic activity, to bring on a period of hyperactivity. Usually at the end of the exposure the division has been completed, and the cells hurried into the resting stage.

5. Only a very short stimulation is necessary to produce this acceleration.

6. Following the phase of acceleration a phase of depression sets in; the end result is to retard greatly the development of the egg.

7. The depression never follows until a complete mitotic cycle has been passed. Thus if the egg is exposed in the first maturation mitosis the depression does not occur until the first cleavage.

8. An egg may be stimulated during the depression phase by a second exposure to the rays, but the new phase of acceleration is less and the depression follows more rapidly.

9. The control, started at the time of exposure, goes more slowly than the experiment during the first two mitoses, but by the time that the twenty-four cell stage is reached the exposed eggs are progressing more slowly that it.

10. It has not been found possible to account for these effects on the basis of rise in temperature, and the nature of the experiment practically eliminates other disturbing factors; therefore, the effects must be regarded as the result of exposure to the X-rays.

11. Analogy suggests that the effect of X-rays on cell divisions may perhaps be due, partly at least, to the effect of the rays on enzymes contained within the cell.

Discussion of the Literature.

Many observations have been made as to the effect of X-rays and radium on growth and rather diverse results have been obtained. Extensive bibliographies in which the previous work has been quite thoroughly reviewed are to be found in the following publications: Warthin, A. S. (*International Clinics*, 1906, 15th series, Vol. IV.); Gager, C. S. (*Memoirs of New York Botanical Garden*, IV., 1908); Bardeen, C. R. (*Jour. of Exp. Zool.*, Vol. IV., 1908 and *Amer. Jour. Anat.*, Vol. XI., 1911). As far as I have been able to discover, few previous observations have been made on the effect of radiation upon the rate of division. Certain other observations have a less direct bearing but are in line with the conclusions here reached. In view of these facts, it seems necessary to discuss only a few of the papers on this subject.

Bacteria and yeasts have generally been found to be inhibited by exposure to radiation if sufficient stimulus to effect them was given. Koernicke, however, states that if the organisms be transferred to fresh unexposed gelatin, they will grow again, and Gager found budding in yeasts to be increased by exposure.

Certain others of Gager's observations on the effect of radium on plants are concerned with the problem of growth. He reports among his results a cessation of cell division, an acceleration of differentiation, a decrease in cell size, and a lack of coördination in histogenesis. Those processes which go to produce senescence are accelerated.

Guillemont compared the action of X-rays and of the beta rays of radium upon plant cells. He obtained a standard for comparison and found that, the flourescent effect of the two being equal, the beta rays were more intense. The characteristic action is a retardation of the growth, when the rays are fairly strong. He determined also the fatal strength and a comparatively weak strength at which the rays perhaps accelerate.

Becquerel likewise found that weak, or short, stimuli had small effect, while longer ones retarded growth. Exposing seeds for a day had little effect upon their power to germinate, but exposure for a week or more inhibited germination. Pollen germination is also inhibited, according to Lapriore, by exposure to X-rays.

Maldiney and Thouvenin, however, early reported that germination of seeds was hastened by exposure to X-rays.

Gager obtained retardation of growth following exposure of seeds under various conditions. The amount of retardation varied directly with the strength of the radiation. Some kinds of seeds, exposed to radium of weak activity, later showed apparent recovery. It has been shown that hydrogen and hydroxyl ions stimulate germination. Gager says with regard to this, "If the radium rays produce ionization in the mineral solutions in the soil then these ions would act as a stimulus to plants growing there, and, under suitable conditions, cause an acceleration of growth. It is not improbable that the results recorded above are due to a combination of both causes, that is, to the direct action of the gamma rays combined with that of ions produced by the rays in the soil-solution."

Gager in his memoir discussed at length the work done previous to 1908 upon both plants and animals. The results upon which there is any very general agreement, he summarizes in the following eight statements:

"1. Radium rays have the power to modify the life-processes of both plants and animals.

"2. Röntgen rays and radium rays produce similar physiological results.

"3. Sensitiveness to these rays varies with the species of either plant or animal.

"4. Younger, and especially embryonic tissues, are more sensitive than those more mature.

"5. With only one or two exceptions, exposure to radium rays has been found to either retard or completely inhibit all cell-activities. The rays may cause irregularities in mitosis.

"6. Experimental evidence for or against the existence of a radiotropic response is conflicting.

"7. Whatever the immediate, internal change produced in the protoplast may be, the result, with animals as well as with plants appears to be more or less profoundly modified by the presence of chlorophyll in the cell.

"8. Radium rays appear to retard the activity of enzymes."

Gager suggests in his final discussion of the results in his memoir (p. 271) that "the rays may operate so as to increase or decrease the amount of energy available for the work" (meaning metabolic processes) "and, lastly, variations in growth may be, either wholly or partly, expressions of the influence of the rays on cell division." In the latter case growth would be an index as to the effect of the rays on the reproductive functions of the cell, and this, it would seem, is highly probable.

He says also, "No one has yet succeeded in accelerating the rate of cell division or in increasing its amount in a given tissue by means of radium rays. The only results recorded are the introduction of irregularities and complete inhibition." After some discussion of this he says further, "Thus we should expect *a priori*, a retardation and finally a complete inhibition of cell division in all tissues exposed to rays of sufficient activity and for a suitable period of time. And this is what has been observed to occur. Theoretically we ought also to be able to accelerate the process by suitable conditions of exposure, but such conditions have not yet been discovered." Whatever the effect of radium, that of X-rays is most positive in regard to its ability to accelerate division, at least under certain conditions.

His final paragraph is also of interest in this connection. He says:

"The broadest, and at the same time the most definite generalization warranted by the work so far done is that the rays of radium act as a stimulus to metabolism. If this stimulus ranges between minimum and optimum points, all metabolic activities, whether constructive or destructive, are accelerated; but if the stimulus increases from the optimum toward the maximum point it becomes an over-stimulus, and all metabolic activities are depressed and finally completely inhibited. Beyond a certain point of over-stimulus recovery is impossible, and death results."

Another statement of the same fact is given on page 157: "The rays of radium act as a stimulus to protoplasm. Retardation of growth following exposure to the rays is an expression of over-stimulation, acceleration of growth indicates stimulation between a minimum and an optimum point."

Protozoa present some variation in their reaction to X-rays. Some are killed but others are very resistant and appear little disturbed. Some are much more susceptible than others. In some cases cytoplasmic and nuclear activities are affected, while in other cases such a process as conjugation goes on apparently unaffected. There is no positive evidence of tropic responses to radiations. Bardeen reports that *Paramœcium* may be exposed as much as twelve hours without disturbing conjugation, or the rate or forms of division.

The effect of radium upon *Ascaris* eggs has been studied by Perthes, by Barlow and Bonney, and by Paula Hertwig. The former found that after exposure the cell divisions became slower and more irregular than the control, and finally gave rise to irregular cell masses or misshapen little worms especially abnormal at the posterior end. The controls, however, gave rise uniformly to active worms. The eggs in the resting and dividing conditions were equally affected, and the degree of exposure is the factor upon which the result depends. Nuclei and in particular the chromatic structures were most injured, while spindle and centrosomes appeared quite normal. The chief effects did not appear at once upon stimulation but only after a certain period of time had elapsed. The results obtained from X-rays were entirely analogous to those from radium.

Barlow and Bonney, studying "the influence of radio-activity

on the division of animal cells," found in *Ascaris* eggs a retardation of the early cell divisions which was followed by death. According to them, a short radiation causes an acceleration of division.

Fräulein Hertwig investigated the effect of radium on *Ascaris* eggs to find evidence as to whether the chromatin and other nuclear structures are directly affected by radiation, as claimed by O. and G. Hertwig, or whether the rays act to break down lecithin and affect the chromatin only indirectly, as held by Schwarz, Schaper and others. Her evidence goes to support the former view. Furthermore, she is not in agreement with all previous work on this form. She finds cytological evidence that even in the first division after exposure the chromatin is affected, although Perthes speaks of what might be regarded as a latent period. She agrees with Barlow and Bonney that exposure causes a retardation, but was unable to secure acceleration of the divisions even with so short an exposure as five minutes. The amount of retardation depends to some extent on the length of radiation, eggs radiated one hour with a given preparation developing farther than those radiated two hours.

Negative results in exposing eggs of sea urchins to X-rays have been reported by both Schwarz and Bardeen, but the recent brilliant studies of Gunther Hertwig as well as the older paper by Bohn on the effect of radium on these forms make it desirable to repeat the experiments with X-rays. Hertwig's results, like those of Fräulein Hertwig, were chiefly concerned with the behavior of nuclei of exposed eggs and his results are convincing along that line. During the course of his experiments he noted that the progress of division in the sea urchin eggs, which had been fertilized with sperm exposed to radium bromide rays, was very greatly retarded even from the first cleavage. At the end of the second day most of these eggs had died, after a decidedly irregular course of development. His most important results do not bear upon the question here under discussion.

Bohn found that an exposure to radium of forty minutes accelerated segmentation in eggs of the sea urchin, although a longer exposure retarded it.

The only previous experiments upon Gasteropods, so far as I am aware, are those of Tur upon the development of eggs of the

snail *Philine* after exposure to radium. He states that in eggs exposed before the first division the segmentation was in no wise delayed by the action of the radium, but that the cleavage as well as gastrula formation was normal. Only later did the effects of radium show themselves. My results do not confirm these observations.

Congdon studied the effect of the beta rays upon several forms. He found that an exposure of twenty-four hours caused a retardation of 31.2 per cent. in the development of the eggs of *Drosophila*. The more intense the radiation the greater the retardation. In these experiments the intensity was measured by the distance of the object from the radium. "Secondary beta radiations (slow electrons) produce a much stronger effect than primary radiations (rapid electrons) of like intensity." He experimented upon Tubularia varying the length of the exposures. Both in *Drosophila* and in the hydranths, he states, "many stimuli which retard or stop growth if of high intensity will accelerate it if they are weak enough." The retardation varies directly as the length of the exposure. "When the fundaments of regenerating *Tubularia* hydranths were exposed to beta radiations from three hundred milligrams of impure radium one thousandth as strong as the pure bromide for periods up to three days in length, the shorter exposures were found to accelerate regeneration and the longer to retard. The degree of retardation increases slowly with lengthening exposure; but the degree of retardation relative to the length of exposure decreased with lengthening exposure."

Again he found that seeds were most sensitive to radiation when the embryos were turned toward the radium. Here also the slower electrons of the beta radiations were more effective relatively than the more rapid.

Zuelzer also reports that insects are affected by exposure to radium.

The vertebrates have served as objects for a large part of the experiments with radium and X-rays. Gilman and Baetjer exposed hen's eggs for ten minutes daily to X-rays. During the first thirty-six hours the development was accelerated. Then there followed a retardation during which the development was greatly altered as well as checked.

Comparable results were obtained by these same investigators working on the eggs of *Amblystoma*. Exposures of fifteen minutes daily first produced a period of acceleration which lasted up to ten days in some embryos, but at the end of four days abnormalities began to manifest themselves. Up to the tenth or eleventh day the exposed eggs were larger than the controls; after that they grew no larger, some became actually smaller, and all were grotesque. The controls on the other hand continually grew larger.

In other eggs which were exposed daily four or five times, but otherwise permitted to develop undisturbed, the tendency to recover and develop normally was noted. This was not a clear result, however, for in less than half of the eggs so exposed was restitution of form affected, and all died after the exposure of the twenty-third day.

The occurrence of a latent period is reported by Schaper as one of the results of exposing eggs of *Rana fusca* and of *Triton* to radium. During the first day of his experiments no departure from the normal course of development was noticed. Following this "latent period," Schaper observed that the development of the embryos was greatly interfered with, marked abnormalities and finally death being produced. The duration of the latent period depends upon the intensity of the radiation and upon the state of development of the embryo at the time of exposure. In nearly all cases it lasted a day, and if older larvæ were used, with relatively short radiation, it might last several days. The course of development was always more or less drawn out, passing into a condition of standstill to be followed at last by death. In general, Schaper found that there were inhibitive effects on cell division, embryonic differentiation, and embryonic growth.

Bardeen has found by exposing either sperm, or eggs before fertilization, or fertilized eggs to radium that abnormalities are produced and he proceeds upon the hypothesis that the nuclei are affected, thus causing the retardation in growth. "Cleavage in most eggs fertilized by exposed sperms seemed to be normal. In several of the experiments it appeared to be slightly more rapid than in the control eggs." In mature eggs which were exposed to X-rays and then fertilized with normal sperm "the early cleavage stage appeared to be normal."

He also says, "It would appear as if the nuclei in mitosis were forced into a resting stage in the spindle by the X-rays."

Finally, O. and G. Hertwig have carried on a thoroughgoing series of studies on the influence of radium upon developing eggs and larvae of amphibians. This set of observations includes first, the nature of the pathological changes due to radiation which are found to occur in the various organ systems and in the body form, and second, the effects of the radiation upon the nuclei and cytoplasm of the various tissues. The evidence is found to support the view that the nuclei are injured directly. In the early stages injury and general retardation of development take place; the effect, however, on the rate of cell division is not discussed, I believe, by either writer.

LITERATURE.

Bardeen, C. R.

'09 Variations in the Susceptibility of Amphibian Ova to X-Rays at Different Stages of Development. Anat. Record, Vol. III.

'11 Further Studies on the Variation in Susceptibility of Amphibian Ova to X-Rays at Different Stages of Development. Amer. Jour. Anat., Vol. XI.

Barlow, Lazarus, W. S. and Bonney, Victor.

'09 The Influence of Radioactivity on the Division of Animal Cells. Archives of Middlesex Hospital, Vol. 15. (Accessible to me only through reviews.)

Becquerel, H.

'01 Sur quelques effets chimiques produits par le rayonnement du radium. C. R. de l'Acad. de Sci., T. 133, p. 709.

Bohn, G.

'03 Influence des rayons du radium sur les oeufs vierges et fécondés, et sur les premiers stades du dévellopment. C. R. de l'Acad. de Sci., T. 136, p. 1085.

Congdon, E. D.

'12 The Influence of Radiations of Radium upon the Embryonic Growth of the Pomace-fly, Drosophila ampelophila, and upon the Regeneration of the Hydroid, Tubularia crocea. Bull. Mus. Comp. Zool., Vol. 53, No. 7.

'12a. A Comparison of the Alterations in the Velocity of Growth of Certain Seedlings through the Action of Rapid and Slow Electrons of the Beta Rays of Radium. Arch. f. Entw-m., Bd. 34.

Conklin, E. G.

'97 Embryology of Crepidula. Jour. of Morph., Vol. 13.

'02 Karyokinesis and Cytokinesis. Jour. Phila. Acad. Nat. Sci., Vol. XII.

'12 Experimental Studies on Nuclear and Cell Division in the Eggs of Crepidula. Jour. Phila. Acad. Nat. Sci., Vol. XV.

Gager, C. Stuart.

'08 Effects of the Rays of Radium on Plants. Mem. N. Y. Bot. Garden, IV.

Gilman, P. K. and Baetjer, F. H.

'04 Some Effects of Roentgen Rays on the Development of Embryos. Amer. Jour. Physiol., X., p. 222.

Guilleminot, H.

'07 Effects comparés des rayons X et des radium sur la cellule végétale. C. R. de l'Acad. de Sci., T. 145, p. 798.

Hertwig, Gunther.

'09 Radiumbestrahlung unbefruchteter Froscheier und ihre Entwicklung nach Befruchtung mit normalen Samen. Arch. f. Mikr. Anat., Bd. LXXVII., Abt. 2.

'11 Das Schicksal des mit Radium bestrahlen Spermachromatins in Seeigelei. Arch. f. Mikros. Anat., Bd. LXXIX., Abt. 2.

Hertwig, Oskar

'09 Die Radiumkrankheiten tierischer Keimzellen. Arch. f. Mikros. Anat., Bd. LXXVII., Abt. 2.

Hertwig, Paula

'09 Durch Radiumbestrahlung hervorgerufene Veränderungen in den Kernteilungsfiguren der Eier von Ascaris megalocephala. Arch. f. Mikros. Anat., Bd. LXXVII., Abt. 2.

Holmes, S. J.

'00 The Early Development of Planorbis. Jour. Morph., Vol. 16.

Koernicke, M.

'04 Ueber die Wirkung von Röntgenstrahlen auf die Keimung und das Wachstum. Berichte der Deutschen bot. Gesellsch., Bd. XXII.

'05 Weitere Untersuchungen über die Wirkung von Röntgen und Radiumstrahlen auf die Pflanzen. Ibid., Bd. XXIII.

Levy, O.

'06 Mikroskopische Untersuchung zur Experimenten über den Einfluss der Radiumstrahlen auf embryonale und regenerative Entwicklung. Arch. f. Entw-m., Bd. XXI.

Maldiney and Thouvenin

'98 De l'influence rayons X sur la germination. Revue gen. de Bot., T. X.

Packard, Charles

'14 The effect of Radium Radiations on the Fertilization of Nereis. Jour. of Exp. Zoöl., Vol. XVI.

Perthes, G.

'04 Versuche über den Einfluss der Röntgenstrahlen und Radiumstrahlen auf die Zellteilung. Deutsch. med. Wochensch., Nr. 17 und 18, Bd. 30.

Schaper, A.

'04 Experimentalle Untersuchungen über den Einfluss der Radiumstrahlen und der Radiumemanation auf embryonale und regenerative Entwicklungsvorgänge. Anat. Anz., Bd. XXV.

Schaudinn, F.

'99 Über den Einfluss der Röntgenstrahlen auf Protozoen. Arch. f. Ges. Physiologie, Bd. LXXVII.

Schwarz, G.

'03 Uber die Wirkung den Radiumstrahlen. Archiv f. Physiol., Bd. 100.

Tur, J.

'09 Sur le développement des oeufs de Philine aperta L. exposés l'action du radium. C. R. de l'Acad. des. Sci., T. 149.

Zuelzer, M.

'05 Ueber die Einwirkung der Radiumstrahlen auf Protozoen. Archiv. f. Protist., Bd. V.

HABITS OF THE LARVA OF BELLURA MELANOPYGA GROTE (LEPIDOPTERA).[1]

PAUL S. WELCH.

Of the great host of *Lepidoptera*, only a few species have invaded the water and acquired aquatic stages in the life history. These few species have solved the problems of maintenance of aquatic life in exceedingly interesting ways, all presenting unique adaptations. The species on which this paper is based is aquatic in the larval stage and rivals the other species of similar habit in the character of its peculiar adaptations.

In 1881 Comstock ('81, pp. 147–149) published a short paper which included Grote's description of a new species, *Arzama melanopyga* (now *Bellura melanopyga*), and a very brief account of the larva of the same, presenting data on its unique habits and adaptations. This seems to be the only published account, the writer having searched in vain for other papers dealing with the habits of this larva. Comstock later ('88, p. 468) made mention of it and Grote ('89, p. 226) called attention to its habits and structures but in neither case were new data presented. The larval habits of a closely allied form, *Sphida obliqua* Wlk., which feeds on *Typha*, are better known and in a few respects resemble those of *Bellura melanopyga*. The following paper includes new data on the unusual habits of the larva and presents the detail of features merely mentioned by Comstock. *Bellura melanopyga* Grt. has been considered by some writers as a synonym of *Bellura gortynoides* Wlk. but the writer follows Hampson ('10, p. 260), who considers *melanopyga* a valid species.

The observations which form the basis of this paper were made by the writer while a member of the staff of instruction at the University of Michigan Biological Station at Douglas Lake, Michigan, during the three sessions of 1911–1913. The abundance of material and the opportunity to make observations at corresponding times during successive seasons made it possible

[1] Contributions from the University of Michigan Biological Station No. 22.

to verify the data and to carry on some phases of the work which otherwise would have been impossible. The observations extended over the months of July and August of each of the three years. Larvæ of *Bellura melanopyga* began to appear during the first week of July and could be found until late in August. They feed on the leaves and burrow lengthwise into the petioles of the yellow waterlily, *Nymphæa americana* (Provancher) Miller and Standley ('12, p. 78). This lily occurs in considerable abundance in the beach pools and the protected bays of Douglas Lake. It also occurs in almost every sphagnum bog in the surrounding territory. This distribution seems to be dependent upon the fact that only at these places are attained the chief conditions which favor their growth, namely, protection from winds and waves, shallow water, and a more or less mucky bottom. *N. americana* was very abundant in one of the inlets (Bessey Creek) and the larvæ of *Bellura melanopyga* were correspondingly numerous, the infestation in 1912 being 90–95 per cent. The percentage of infestation in the beach pools and in the protected bays was much lower, not exceeding 25 per cent. This was probably due to the fact that Bessey Creek is much better protected from wind and waves, factors which are unfavorable to the very young larvæ. The lilies growing in the bogs of the surrounding region showed no signs of infestation.

Feeding Habits.

The feeding habits of this larva were studied in considerable detail. These activities vary considerably according to the age of the larva. Two fairly well-defined periods can be recognized (1) a very early period, the *leaf-feeding period*, or the *mining period*, which lasts approximately throughout the first two stadia, and (2) the later period, the *petiole period*, which includes the remainder of the larval existence.

The Leaf-Feeding or Mining Period.—The eggs have not been observed but it is evident that they must of necessity be laid somewhere on the leaf since, as will be shown in a later discussion, the very young larvæ are not efficient swimmers and were often found in places where it would have been impossible for them to get had the eggs been laid elsewhere. Furthermore, at the

beginning of the season it was no uncommon thing to find twenty or more very young larvæ, 3–4 mm. in length, on a single leaf. The presence of so many very young larvæ on one leaf and the absence of larvæ from a contiguous leaf is taken as evidence that the eggs were laid somewhere about the infested leaf.

During the mining period the larva works on the upper side of the leaf. It usually cuts a somewhat circular hole, slightly larger than itself, through the upper epidermis and penetrates into the parenchyma, thus becoming a *miner*. There is no regularity in the shape of the mine. Sometimes it appears as a winding tunnel with a diameter about twice that of the larva; sometimes it is digitate in appearance; and sometimes it resembles a "blotch mine." The area included in each may vary to considerable extent. Holes through the epidermis other than the original entrance may occur anywhere throughout the length of the mine. Mines are easily detected on the surface of a leaf since they soon become whitish in appearance, due to the removal of the chlorophyll-bearing tissue. They may occur anywhere on the leaf. Leaves were sometimes found in which the mines appeared to extend either towards the midrib or towards the junction of the midrib with the petiole but an examination of a large number of infested leaves leads the writer to believe that no importance can be attached to these cases.

The initial entrance to a mine is usually surrounded by excrement and a small quantity of finely masticated leaf tissue. Microscopical examination shows that the latter is composed of the broken fragments of the epidermal cells and numbers of the peculiar *idioblasts* which are so common in the tissues of the yellow waterlily. Examination also shows that the fragments of the epidermal cells have not undergone digestion and furnishes evidence for believing that the larvæ never use the epidermal tissue as food but simply remove it with the mandibles. The excrement is greenish when first voided but gradually becomes lighter in color until finally all color is completely lost. It is voided in the form of small, somewhat oblong, uniform masses which tend to remain together, forming chains of varying lengths. Microscopical examination shows that it is composed of two kinds of elements: (1) idioblasts, which form as much as 60 per cent. of

the excrement and show no evidence of being affected by digestion, and (2) greenish, unorganized matter, which represents the remains of the digested parenchymal tissue.

Even in the very early stages the larvæ are active feeders. Larvæ, 5½ mm. long, constructed mines 14 mm. long in less than 20 hours. Larvæ, 7 mm. long, when transferred to new lily leaves completely buried themselves in four hours. Usually but one larva occupies a mine. Several instances were observed, however, in which two were occupying the same mine and in one heavily infested leaf eight larvæ of about the same size were found in a single, large mine. The formation of the mine is due primarily to the fact that the larva apparently uses only the parenchyma as food and must get under the epidermis in order to get it. However the mine has an indirect value since it furnishes a protection for the larva during the early and more helpless stages.

Several days after the mine has been formed the upper and lower epidermis bounding it begin to disintegrate, untimately leaving an ugly hole in the leaf and, in badly infested leaves, producing numerous perforations. The effect on the plant is evident. Not only is the leaf disfigured but in proportion to the number of holes present the leaf surface is also reduced. Many cases were observed in which the infestation was great enough to cause the death of the entire leaf.

In spite of the fact that in the Douglas Lake region a goodly number of other insects affect the yellow waterlily some of which also produce holes and burrows in the leaves, it is not difficult to identify the work of young *Bellura* caterpillars. Certain beetles (*Donacia*) make holes through the leaves in order to deposit eggs but these are distinguished by the circular shape of the holes, and by the rows of eggs or the remains of the eggs on the lower side while the holes made by the larvæ of *Bellura melanopyga* are usually irregular and bear no eggs on the lower margin. The larva of another beetle (*Galerucella nymphæa*) works on the upper side of the yellow waterlily leaf but instead of making mines it produces irregular trenches on the surface by eating away the epidermis, giving the surface a brownish, etched appearance.

The Petiole Period.—This period begins when the larva quits

the mining habit and begins to burrow lengthwise down the petiole. It is initiated when the larva locates either the midrib, or the junction of the leaf with the petiole, and from this time the feeding activities are confined almost entirely to the petiole. The latter is usually reached in one of two ways: (1) the larva burrows through the leaf substance or wanders on the surface until it reaches the midrib, whereupon it bores into it, tunneling towards the attachment of the petiole, or (2) the larva burrows or eats through the substance of the leaf or wanders on the surface until it reaches the attachment of the petiole where it then begins to excavate. At about this time the larva has attained such a size that mines can no longer be made but instead a broad slit in the leaf is produced. This slit (Figs. 3–5) is usually a trifle wider than the diameter of the larva producing it and almost invariably extends directly towards the midrib or its junction with the petiole. Sometimes a number of these slits are made before the larva constructs one which reaches into the substance of the midrib but all extend in the same general direction. If the midrib is reached first the larva bores lengthwise into it and towards the junction of the leaf with the petiole. Only very rarely were larvæ found boring in the opposite direction. The work in the midrib is merely preparatory to the work in the petiole, being only a means of getting into the upper end of the latter. It is not possible at present to account for the ability of the larva to recognize the position of the midrib or the petiole from any position on the surface of the leaf, and to distinguish between the apex of the midrib and the attachment of the petiole.

As will be shown later active dissemination of the larvæ begins at the initiation of the petiole period. Many instances were observed in which a larva had come to a new leaf and with surprising exactness had gone directly to the junction of leaf and petiole and had burrowed into it. Other similar instances were observed in which the larva on reaching a new leaf had cut a preliminary slit through the leaf leading directly to the top of the petiole. This ability to work directly from the periphery of a new leaf to the region of the petiole is especially marked in larvæ over 3 cm. in length. These facts were made apparent many times by field experiments in which larvæ 4 cm. in length

were removed from their burrows and placed on other leaves; also whenever it was necessary to restock the large tank, which served as the aquarium, by introducing larvæ from the field. In about 40 per cent. of these cases the larvæ bored into the petiole without any preliminary cutting of the leaf while others cut slits leading directly towards the attachment of the petiole. With the beginning of the petiole period comes the restriction of a single larva to each leaf.

Length of the Burrows.—The length of the burrow in the petiole varies according to the time it has been inhabited, and to some extent according to the size of the larva. Burrows were frequently two feet long and occasionally longer. Sometimes the burrow extended down to the rootstalk but no instance was observed in the field in which the burrow extended into it. There seems to be no reason why this should not occur since it will be shown later that the substance of the rootstalk can be used as food.

Deserted Burrows.—Burrows of various lengths were often found which had been constructed and then deserted without apparent cause. These burrows ultimately collapsed to some extent and became brownish on the inside. Usually they were inhabited by other aquatic animals, such as gyrinid larvæ, crustaceans (amphipods), chironomid larvæ, small aquatic coleoptera, and small leeches.

Formation of New Burrows.—In order to observe the initial steps in the production of a new burrow, larvæ 4½ cm. in length were removed from the petioles and placed in the aquarium on fresh, uninfested leaves. Some took to the leaves and began boring from the upper side of the leaf into the petiole while others attacked petioles which happened to be nearly horizontal and only partly under water. The larva began excavation by biting into the tissue until the mouth was full; then the head was withdrawn and the mass expelled at the margin of the hole. This was continued until the head was buried after which time the larva ate the tissue removed. Microscopical examination of the initial material deposited around the margin of the hole showed that it was merely pulverized leaf tissue.

Other Points of Attack.—Occasionally peduncles were found both in the field and in the aquarium which had been attacked.

In all cases the initial point of attack was at the top of the flower. Both opened and unopened flowers were affected. Floating rootstalks in the aquarium were always attacked and, if left for any length of time, were ultimately excavated through and through. This utilization of the substance of the rootstalk was not always caused by famine. A number of specimens were kept under conditions where rootstalk substance alone could be secured as food and apparently thrived on it. It thus appears that such materials may serve as food for these larvæ.

It sometimes happened that a fall in the level of the water caused portions of the long petioles to be bent above the surface of the water, or at least to lie on the surface. Whenever larvæ discovered these emergent petioles they attacked them, making an entrance through the side of the petiole.

Excrement.—In the field the best mark of recognition of the work of these larvæ is the heap of excrement which accumulates around the margin of the burrow on the upper side of the leaf. As would be expected the quantity depends upon the length of occupancy and the activity of the larva. In August it was a common thing to find hundred of leaves with conspicuous heaps of excreta around the hole on the upper surface.

The excrement is always deposited outside of the burrow regardless of the relation of its opening to the level of the water. Since the position of the larva is such that the posterior end is towards the leaf, the excrement is always deposited by thrusting the terminal somites out through the entrance of the burrow. That the level of the water has nothing to do with determining the place of deposition of excrement is shown by the following observations: (1) In floating, partially submerged rootstalks which had been tunneled by the larvæ it often happened that much of the upper part of the burrow contained no water but invariably all of the waste matter was deposited on the outside around the entrance. (2) Instances were observed where the entrance to the burrow was submerged for about an inch, due to a raising of the level of the water. The waste matter was always deposited at the entrance of the burrow and under water.

Microscopical examination of the excrement at all stages of the petiole period showed a composition similar to that described for

the leaf-feeding period. The percentage of idioblasts was large and they showed no indication of having been affected by the digestive process. The majority of the idioblasts were intact. Some showed signs of fragmentation, due to the initial action of the mandibles when the plant tissue was removed. Examination of the excrement also showed that, except for a short time when the larva is cutting away the epidermis and surrounding tissue in starting a new burrow, all of the material which is excavated passes through the alimentary canal of the larva and is subjected to the digestive process. Thus it is evident that the length of a burrow is a criterion of the amount of food material which has been taken from the plant. The larva is a voracious feeder, eating during both day and night, and large quantities of plant tissue are consumed.

Effect on the Food Plant.—The effect of the larva is such that infested plants are doomed. During the latter part of July and early August hundreds of leaves showed incisions converging towards the midrib and more especially towards the attachment of the petiole. Many leaves turned yellow and disintegrated, due to one or both of two causes: (1) eating in the region of the attachment of the petiole almost or entirely severed the connection of the leaf with the petiole; (2) the removal of the greater part of the substance of the petiole and the occasional eating through one side produced a more or less complete separation of the leaf from the rootstalk.

It thus appears that these larvæ produce havoc in the beds of *Nymphæa americana* and are a serious enemy since they not only disfigure the leaves but also actually cause the destruction of the same. They also destroy the flowers to some extent. In fact, of the goodly number of insects found on *N. americana* in the Douglas Lake region, the *Bellura* larvæ are usually the worst enemy. Fortunately the rootstalks seem to suffer but little from their attacks.

Other Plants as Food.—Observations and experiments showed that under normal conditions these larvæ use only the yellow waterlily as food. In the field none were found feeding on the white waterlily (*Castalia odorata*) notwithstanding the fact that in many cases *Nymphæa americana* and *Castalia odorata* occurred

in the same locality, frequently intermingling and contiguous. In the region studied *Castalia odorata* is surprisingly exempt from insect attacks. Beutenmüller ('02, p. 440) states that, in the vicinity of New York City, the caterpillar of *Bellura melanopyga* "bores in the leaf-stalks of the common white pond lily and yellow pond lily." Assuming that the "common white pond lily" referred to above is *Castalia odorata*, it appears that the larva may work normally on it in certain other localities. However, the evidence collected in the Douglas Lake region is entirely contradictory.

Experiments were carried on at the laboratory with the view of determining whether or not these larvæ could be forced to use other food. The following are typical experiments and results: *Experiment 1.* Two larvæ, each 6 mm. in length, were transferred to a watch glass containing a piece of leaf of the white waterlily (*Castalia odorata*) which was kept moist. During the first eighteen hours the larvæ wandered restlessly about, then finally began working on the white waterlily leaf and at the end of four hours one had completely buried itself and the other was making similar headway. Fresh pieces of the white waterlily leaf were used to replace the eaten ones from time to time and these larvæ were kept thus for twelve days. At the end of that time both were alive, apparently in healthy condition and one ecdysis had occurred. The results show that it is possible for the larvæ to use the white waterlily as food when forced to do so by the absence of the normal food. *Experiment 2.* Both yellow and white waterlilies were placed in the same aquarium which was stocked with several larvæ, 4 cm. in length. The larvæ attacked the yellow waterlilies and worked on them continuously. Ultimately the food plants were consumed and a famine was allowed to occur. After a lapse of several hours only one case was observed where larvæ were attempting to tunnel into the petiole of one of the white waterlilies and it was finally abandoned, the larvæ wandering restlessly about the aquarium. *Experiment 3.* Larvæ were placed in covered dishes containing *Potamogeton natans* and *Sagittaria* sp., plants which occurred in abundance in the same habitat with the waterlilies, and were left thus for several days. The tissues of these plants were invariably and constantly refused.

Respiration.

One of the problems which confront an insect possessing an aquatic stage is that of securing the requisite amount of oxygen. Those few genera of *Lepidoptera* which are unique in having aquatic larvæ have solved the problem in one of two ways: (1) by the utilization of the dissolved oxygen, either by means of gills or through cutaneous respiration, or (2) by making periodic trips to the surface in order to secure the oxygen from the air. Each of these adaptations calls for distinctly differentiated structures and each is accompanied by interesting habits and activities. *Bellura melanopyga* has solved the oxygen problem by a peculiar development of the spiracles and by making *periodic trips to the surface.* A careful study has been made of this adaptation and the results will be given in some detail.

Respiratory Apparatus.—Paired spiracles are present on I, IV, V, VI, VII, VIII, IX, X, and XI. All, exclusive of the pair on XI, are similar in size, are elliptical in shape, are lateral in position, and have the long axis vertical. Somites I–X are similar but XI–XIII form the chief structural differentiation accompanying this aquatic habit. XII is very short, being only about one-fourth as long as XI, and its dorsal surface is depressed considerably below the corresponding surface of XI. The dorsal, posterior margin of XI bears two large, elliptical spiracles, one on each side of the median line. They are placed obliquely, the long axis being inclined about forty-five degrees from the vertical. They are at the posterior termini of the lateral, longitudinal tracheal trunks, opening directly into them. All other spiracles have short branches leading into the longitudinal trunks. Each of the terminal spiracles on XI opens into a somewhat enlarged region of its respective longitudinal trachea which probably serves as a reservoir for the supply of air which is carried below the surface by the larva on its downward trips. XIII is somewhat larger than XII and forms the cauda. Its dorsum is on the same level with that of XII and the lateral margins converge caudad.

The Leaf-Feeding Period.—During the leaf-feeding period respiration is carried on in the same way as in terrestrial lepidopterous larvæ. Each larva is working in a mine in the leaf but

the entrance hole and other openings which usually occur are sufficient to provide the necessary air.

The Petiole Period.—When the larva deserts the leaf and becomes a borer in the petiole new conditions are encountered and new provisions must be made. The leaves of *Nymphæa americana* are never normally held above the surface of the water by the petiole as in the case of *N. advena* but the emergent leaves float on the surface. This means that the burrow in the petiole is filled with water and that the larva is submerged. When the length of the burrow increases to such an extent that it is longer than the body of the larva which is making it, the latter is compelled to resort to other means of getting air. To do this the larva makes *periodic trips* to the surface where the fresh air is drawn into the tracheal system in sufficient quantity to allow a sojourn of several minutes under water at the bottom of the burrow.

As stated previously, the larva lies in the petiole with the posterior end towards the top of the burrow. This position eliminates the necessity for the larva to come out of the burrow when taking air, and to turn around when starting on its trip to the top. When at the bottom of the burrow the larva feeds (or in some cases merely rests) until the need for air stimulates it to return to the surface. Then it *backs up* to the top of the burrow, stopping when the large pair of spiracles on the posterior margin of XI is just pushed above the surface film. It remains in that position until sufficient air has been taken into the trachea to permit a return to the bottom of the burrow again. This alternate sequence of feeding and breathing goes on continuously so long as the larva remains in the burrow. In this form of respiration it appears that the other spiracles are not needed since only the posterior pair is pushed above water.

Frequency of Trips to the Surface.—A number of observations were made on various larvæ in order to determine what is the normal period of time spent under the water and likewise the normal period spent at the top. The records were made by means of a stop-watch and the observations on each larva were carried on long enough to secure data showing the range of variation in each case. The average of 128 observations involv-

ing 13 different larvæ, 4–6 cm. long, showed that the interval spent below the water was 2 minutes and 57 seconds, and the time interval spent at the top was 1 minute and 21 seconds. The maximum period of time voluntarily spent below was 13 minutes while the minimum was 20 seconds. The maximum period at the top was 6 minutes and 40 seconds, and the minimum was 5 seconds. Comstock ('81, p. 148) states that he observed larvæ of this species remain under water for a half hour but in the large number of observations made in this connection none remained below over 13 minutes.

In connection with the observations on the frequency of trips to the surface, the question arose as to whether there was any correlation between the length of the burrow and the length of time between appearances at the top. In order to determine this point observations were made on a number of larvæ from day to day as they increased the length of their burrows. Results show that the length of the burrow does not in any way determine the time spent below the water. In fact, they show that the intervals are approximately the same when the burrow is but 6 cm. long as when it is over 20 cm. in length. From an examination of all results secured in connection with this study, the writer is convinced that the length of the periods spent under water is purely a matter of supply and demand of oxygen, that it is dependent upon the amount of air which the larva draws into the trachea when at the top and the activity of the larva in the process of feeding at the bottom of the burrow.

Resistance to the Lack of Oxygen.—Experiments were carried on to determine how long larvæ can live under water, when forced to do so, without renewal of the air supply. The entrances to the burrows were plugged in such a way that the inmates could not get air at the top, and the time recorded. These petioles were visited at regular intervals and the condition of the larvæ noted. Larvæ were also placed in tubes of water which were corked so that no air spaces were left at the top, and the condition of the specimens carefully observed. These experiments showed that death usually occurred after an interval of about two hours. After 15 or 20 minutes of continued submergence larvæ began to show the first signs of uneasiness and evidences of weakness began to appear at the end of an hour.

In this connection another set of experiments was carried on in which the infested lily leaves were staked down under water to a depth of six or seven inches and the behavior of the larvæ under these new conditions noted. Each larva came to the top of the burrow and gradually pushed the posterior end out through the entrance, continually searching in a random fashion for the surface film. Ultimately it loosened its hold, came to the surface, and swam about until it came to another lily leaf. In some cases larvæ lived for 45 minutes under water before they would release the hold and come to the top.

Expired Air.—After a larva has been at the bottom of a burrow for a brief interval one or two bubbles usually rise to the top. This happens commonly at the end of the period below and just before the beginning of the trip to the top. These bubbles probably represent, in part at least, expired air which is expelled from the tracheæ.

The Breathing Position.—Except when the larva voluntarily leaves the burrow, it only tips the surface with the posterior end when taking air. Normally only the dorsal surface of XII and XIII and just enough of the posterior margin of XI to include the large dorsal spiracles is above the surface film, the other portions of the body remaining under water. When the larva comes to rest at the breathing position the terminal spiracles pull down the surface film, forming a small conical depression. When in this position the larva is very sensitive to surface disturbances and responds quickly to them by retreating into the burrow. Thus in making observations on the breathing activities it was very necessary to avoid making even the smallest ripples or swells. Very tiny jars on the leaf produced the same response. The alighting of a small insect on the same lily leaf was sufficient to cause the larva to dodge below. It is very probable that this response is responsible for the avoidance of many attacks by predaceous enemies.

Breathing Movements.—If a larva in the breathing position be watched carefully it will be noted that certain rhythmic movements are performed. These movements occur at intervals of from two to three seconds and appear to consist of an alternate contraction and expansion of the exposed region.

Extrusion of Excrement.—The voiding of excrement occurs in connection with the respiratory trips to the surface but since these trips are the direct effect of the demand for oxygen the extrusion of wastes is only an accompanying feature. The ratio of the number of respiratory trips to the number of extrusions is variable, depending upon the feeding activity of the larva. The following records will serve to illustrate the variation:

Larva no. 1.....2 extrusions in 14 trips to surface.
Larva no. 2.....1 extrusion in 11 trips to surface.
Larva no. 7.....1 extrusion in 41 trips to surface.

Locomotion.

Dissemination is accomplished in three distinct ways, viz., swimming, floating, and crawling.

Swimming.—The larva usually swims on the surface although when forced to do so it can swim to a limited extent below the surface. The specific gravity of the larva in all stages is very slightly less than that of water, thus making it easy to remain at the surface. Furthermore, the larva at all stages has an oily surface which prevents a "wetting" of the exterior and constitutes another means of remaining on the surface. This form of locomotion is accomplished by means of a series of rather vigorous horizontal undulations in the execution of which the whole body takes a part. The caterpillar lies in a trough in the surface which partially surrounds it and is propelled by the characteristic sinuous movements. The efficiency of this kind of locomotion varies with the age of the larva and the amount of disturbance of the surface of the water. Very young larvæ do not progress very rapidly but the full-grown ones are efficient swimmers, showing noteworthy speed and endurance. Larvæ, 4 cm. in length, were removed from their burrows and placed in the open water in a protected bay where the surface was perfectly quiet. They immediately began to swim and records were made of the rate of progression. In one instance a larva made a continuous swim of 250 feet in 19 minutes and at the end of the journey showed little or no signs of weakness. A number of similar records were made with similar results. Ripples, diminutive waves, and currents interfere seriously with this form of loco-

motion, even with full-grown larvæ, and little or no headway can be made against them. Owing to the fact that the situations which are favorable for the growth of their food plant are characterized by quiet water, the interference with dissemination due to surface disturbances is not great.

The advantage to the larva of this surface swimming is obvious. It must have free air and many of the journeys which it makes require a longer time than it can remain under water but the ability to swim on the surface makes it possible to secure air with ease.

When larvæ are placed beneath the surface of the water and released they immediately orient themselves in such a way that the head is towards the surface and, by means of the undulatory movements of the body, swim diagonally upward, continuing the effort until the surface is reached. Swimming below the surface is much less efficient than on the surface but it is usually effectual in assisting the larva in getting back to the top. When submerged they appear to lack the ability to do effective climbing on stems and other objects with which they come in contact.

When swimming on the surface the larva shows a strong tendency to climb upon any object which comes in its way and in case the object is immovable, has a smooth upper surface, is above the surface of the water, and broad enough to support the greater part of the length of the body, it will usually come to rest for a time. In cases where the object is very limited in size, as for example a stick, twig, or stem, the larva usually climbs over it and continues the journey. Larvæ may desert the leaves and voluntarily take to the water. When once in the water the swimming movements are usually kept up continuously until another flat, emergent object is reached. When a larva deserts one lily leaf the search for another food plant is entirely a random one and the contact with another leaf seems to be purely accidental. It merely keeps on swimming until it happens to choose a course which leads to a leaf. The larva apparently has no means of recognizing the presence of a food plant except when in contact with it. The writer observed instances where larvæ swam aimlessly about for a half hour without finding a food plant, having passed several times within a centimeter of a lily leaf.

The larvæ under observation showed a definite response to currents of low rapidity. When placed in the open water of a stream having a current of 10 feet per minute they almost invariably swam down stream. This accounts for the prevalence, at the mouth of Bessey Creek, of infested leaves showing attack only at the junction of the petiole with the leaf—an indication of the work of the older larvæ.

Floating.—Instances were observed where larvæ were transported from place to place by floating down stream on detached leaves and other floating objects. Occasionally a swimming larva became passive and, since it remained on the surface, it was carried along with the current.

Crawling.—Change of position on the leaf is accomplished by crawling and, in case lily leaves are contiguous, means is thus provided for the passage from one leaf to another. When in search of a new leaf the larva usually explores the periphery of the leaf, taking to the water and swimming only when it is not possible to reach another leaf from the supporting one. The explorations at the edge of the leaf consist in projecting one-third to one-half of the body over the edge and swinging it about as if seeking another support. If nothing is within reach, the body is withdrawn and the same performance repeated at another place. If, however, an object is within reach the larva immediately crawls upon it.

In cases where larvæ were deserting the old leaves it was not always possible to account for the departure. The two most common incentives apparently were (1) the presence of other larvæ on the same leaf, since only one larva can occupy the burrow in the petiole, and (2) the deterioration of the lily leaf and petiole which results in an unfitting of the plant for the larva.

Enemies.

These larvæ were eagerly snapped up by sunfish whenever there was opportunity. They were comparatively safe in the burrows but whenever they left the lily leaves and swam in open water the mortality was often high. Specimens removed from the petioles and thrown out into open water were soon discovered and captured. The undulatory swimming motion appeared to be

fatally effectual in attracting the attention of fish. A number of instances were observed in which specimens thrown out were not molested so long as they remained motionless but unfortunately for them they remain quiet for only short intervals, often not exceeding 60 seconds, and soon after swimming began were snapped up. Fish of all sizes appeared to feed upon them. The larger fish always secured the prey at the first dash but small fish were observed to make three or four attempts before the full-grown larva could be secured.

The large water strider (*Gerris* sp.), common in the Douglas Lake region, was observed to attack the larvæ when they happened to be on the surface of the leaves. The ultimate effects of such an attack were not observed since in every case the larva attacked happened to be under observation for other data and the striders were driven off.

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LITERATURE CITED.

Beutenmüller, W.

'02 Descriptive Catalogue of the Noctuidæ found within Fifty Miles of New York City. Part II. Bull. Am. Mus. Nat. Hist., 16: 413–458. 4 pls.

Comstock, J. H.

'81 An Aquatic Noctuid Larva. Papilio, 1: 147–149.

'88 Aquatic Lepidopterous Larvæ. Am. Nat., 22: 468–469.

Grote, A. R.

'89 The Noctuidæ of North America and Europe compared. (Fourth Paper). Can. Ent., 21: 226–230.

Hampson, G.

'10 Catalogue of the Lepidoptera Phalænæ in the British Museum, 9: 260.

Miller, G. S. and Standley, P. C.

'12 The North American Species of Nymphæa. Contr. U. S. Nat. Herbarium, 16: 63–108. 12 pls., 40 figs.

EXPLANATION OF PLATE.

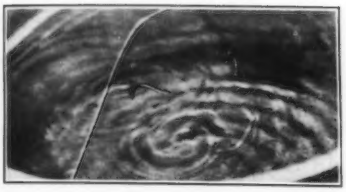
FIG. 1. Photograph of a nearly full-grown larva of *Bellura melanopyga* swimming on the surface of the water in the aquarium. The undulations of the body in performing the swimming movements are evident.

FIG. 2. Photograph of the same larva taken a few seconds later.

FIG. 3. Leaf of *Nymphæa americana* showing the character of the work of approximately mature larvæ. The radiating slits represent the work of the larvæ preliminary to the excavation of the petiole. Note the severe injury to the leaf in the region of the petiole. Leaves are sometimes almost completely severed from the petiole in this way.

FIG. 4. Leaves of *Nymphæa americana* showing the work of half- to full-grown larvæ and the characteristic form of the injury.

FIG. 5. Leaves of *Nymphæa americana* infested by half- to full-grown larvæ. The leaf near the center of the figure shows the absence of preliminary cutting in the form of radiating slits. Note on the same leaf the heap of excrement about the hole at the junction of leaf and petiole. This hole is the entrance to the burrow in the petiole.



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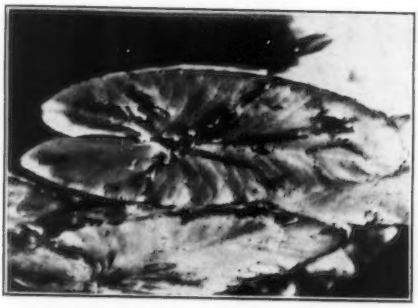

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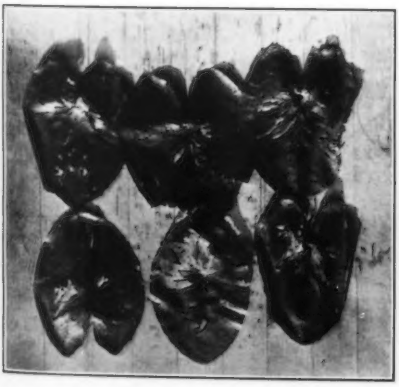

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FERTILIZATION IN THE PARASITIC COPEPOD, LERNÆOPODA EDWARDSII OLSSON.[1]

NATHAN FASTEN.

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General Remarks.

Wright (1882), in discussing reproduction in the parasitic copepod *Achtheres micropropteri* deprecates the fact that so little is known of the exact method of fertilization in the parasitic copepods, and adds that "a thorough examination of the male reproductive apparatus of the Lernæopodidæ is very desirable for the purpose of elucidating the formation of the spermatophores in the parasitic Copepoda." Since Wright's work no one has published any results along this line. It, therefore, seemed advisable to investigate this problem a little more fully.

The following studies were made on the parasitic copepod *Lernæopoda edwardsii* Olsson which infests the gills of the brook trout *Salvelinus fontinalis*. The writer, while in the service of the Wisconsin Fish Commission, during the summer of 1912, secured a lot of fine stages of this organism in copulation, from the time the male attaches itself to the female until the actual fixation of the spermatophores near the genital pores of the female's body. Many mature males and females were also secured.

The material was fixed in a 5 per cent. solution of corrosive-acetic for eighteen hours and was then washed in running water for a day. Selected stages were stained in toto in borax-carmine for thirty-six hours, and the excess of stain was removed in water or in very weak acid-alcohol. The material was next

[1] From the Zoölogical Laboratories, University of Wisconsin.

passed through the various grades of alcohol, being left in each for about ten hours, and after being cleared in xylol was finally mounted in balsam.

Many mature males, females, and copulating individuals were also imbedded in paraffine for sectioning. These were cut into frontal, sagittal and transverse sections, ranging from 3–6 μ in thickness, and stained either in Delafield's hæmatoxylin and eosin or in Heidenhain's iron-alum hæmatoxylin and acid fuchsin. The latter stains gave the finest results and were utilized almost exclusively.

The Male Reproductive Organs.

The male reproductive organs of *Lernæopoda edwardsii* are, in the main, similar to those of the free living copepods, as described by Gruber (1879). They are paired, and are located in the posterior half of the body (Figs. 1–2, *t.*, *v.d.*, and *sp.*). These organs lie in the space between the intestine and the lateral body wall, and consist of three main parts: (*a*) the testis (Figs. 1–2, *t*), (*b*) the coiled vas deferens (*v.d.*), and (*c*) the spermatophores (*sp.*). The testis is sac-like in appearance, with its anterior end projecting into a tube-like structure that ends blindly. When a section of the testis is examined under the microscope, three distinct zones can be recognized which correspond very closely to the three zones in the testis of *Diaptomus* sp., described by Ishikawa (1891). The anterior portion is the formative zone, and consists of spermatogonial stages. The middle region is the zone of growing spermatogonial cells, and lastly, the posterior region represents the maturation zone. Here the testis is in active proliferation and is filled with great numbers of spermatozoa. Each testis unites with the vas deferens posteriorly.

The vas deferens is an elongated coiled structure composed of three parts. The first portion of the vas deferens (Figs. 3–4, *v.d. 1*), lies nearest the lateral body wall of the copepod, and receives the spermatozoa from the testis. The second branch (*v.d. 2*), is a thin, tube-like structure that makes its way from the lower margin of the preceding portion (*v.d. 1*) and then coils up diagonally behind it, to a point slightly above the terminal region of the testis. This is best seen in Fig. 4. It then coils forward as the third lobe of the vas deferens (*v.d. 3*). This runs along

posteriorly to a slit-like opening located in the extreme lower margin of the male's body. This opening is the ejaculatory pore (Figs. 3–6, *e.p.*), through which the spermatophore is extruded.

Each spermatophore (Figs. 1–6, *sp.*) originates in the third branch of the vas deferens. It is pear-shaped in form, and in the living organism is of a pale yellowish color. In Figs. 5–7 the structure can best be studied. The spermatogonial pouch, filled with spermatozoa (*sz.*), is enclosed by an outer wall of chitin (*ch.*). Immediately behind this there is a thin layer of cement-like substance (*c.*), that stains intensely black in Heidenhain's iron-alum hæmatoxylin. In the center of the spermatophore is a spherical pouch filled with a similar cement (*c.*) as is evident from the similarity of its staining reaction. This cement substance of the spermatophore appears to be continuous with the small sphere of cement found in the loop-like enlargement of the vas deferens (see Fig. 3, *v.d. 3*).

When the spermatozoa are mature, they are discharged into the first branch of the vas deferens. From here they pass into the second lobe, and finally, they migrate into the canal situated in the center of the third part of the vas deferens. This is shown in Fig. 5, where the canal (*can.*) conducts the spermatozoa into the spermatophoral pouch, located between the central sphere of cement and the outer cement wall. Here the sperm (Figs. 6–7, *sz.*) are stored until copulation occurs.

The Female Reproductive Organs.

The mature female is much bigger than the male, being about three times as long as the latter. Fig. 1 shows this size difference. The sex organs of the female lie dorso-laterally within the abdomen, between the intestine and the body wall. The ovaries (Figs. 1 and 9, *ov.*), are paired in character, and each gives rise to a slender oviduct (Figs. 1 and 9, *od.*), which makes its way around the intestine to the posterior portion of the body, where it opens to the exterior. Both oviducts unite slightly above their terminations into a broad pouch that later becomes the spermatheca and its accessories (Figs. 9 and 11, *s.*). From this pouch, two slender tubes are developed that open posteriorly through the genital pores (Figs. 1 and 8, *g.p.*). Also, within each oviduct,

a small, spiral gland can be distinguished (Figs. 1 and 9, *c. g.*), which, under the microscope shows strong powers of refracting light. These structures later develop into the cement glands of the adult (Figs. 10–11, *c.g.*).

Fertilization.

About two and a half or three weeks after the attachment of *Lernæopoda edwardsii* to the brook-trout, the copeopod is ready to copulate. In two earlier papers (Fasten, 1912 and 1913), the attachment of the parasite to the host was discussed. As already stated, the mature male is very much smaller than the female. In order to fertilize the female, the male must release his hold on the gill of the brook-trout, and attach himself to the lower extremity of the female's body, in the neighborhood of the genital pores. This is accomplished in the following manner. When mature, the male, though still attached, makes circling movements with his body, thus coming in contact with a female. As soon as this occurs, the male clasps her with his maxillipeds, and at the same time, withdraws his second maxillæ from the filament of attachment to the gill. Then he moves towards the female's genital pores, and places himself in position for fertilization. The position assumed is shown in Fig. 1. These observations are similar to those made by Wilson (1911), on the copepod *Achtheres ambloplitis* Kellicott, infecting the rock bass.

When once in position, the male brings forward the posterior region of his body to a position near the genital pores. The spermatophores are then ejected through the ejaculatory pores, and by the aid of his free maxillae the male attaches them near the genital openings of the female (see Fig. 8). The cement found in the spermatophores is the substance that makes them adhere tightly. As soon as they are attached, the spermatozoa wander through the genital pores and are conducted into the spermatheca, where they are stored until the eggs are mature for fertilization. When the spermatozoa have all migrated into the spermatheca, the spermatophores collapse, and become transparent, shell-like, yellowish spheres (Figs. 9–10, *sp.*).

The female may be fertilized more than once. As many as six spermatophores were found clinging to the genital pores of

some of the females, showing that these females have, in all probability, been fertilized three times. After fertilization, the male drops off from the female's body and dies. The female however, grows enormously in size. During this growth period, the ovaries develop and produce a great many eggs. The cement gland also develops completely, and from each side of the extreme lower margin of the abdomen, an egg-sac grows out. The oviducts communicate with these egg-sacs directly. Figure 10 shows the abdomen of an adult female, with the eggs (*o.*), the oviducts (*od.*), the cement glands (*c.g.*), the spermatheca (*s*)., the spermatophores (*sp.*), and the egg-sacs (*e.s.*).

The eggs are discharged into the oviducts, and are then passed down posteriorly, where they are fertilized by the spermatozoa which are stored in the spermatheca. Then they are coated with a layer of cement from the cement glands, and finally pass into the egg-sacs, where they develop into larvæ. The cement coating hardens into one of the egg covers. Fig. 11 shows the relation of the spermatheca (*s.*) to the oviducts (*od.*). The spermatheca is filled with great numbers of ripe spermatozoa.

These observations on the structure of the reproductive apparatus of the adult female corroborate those of Miculicich (1905), who worked with Lernæopodidæ of the genus *Brachiella*.

I wish to express my thanks to Professors M. F. Guyer and A. S. Pearse for reading this paper and for their helpful suggestions.

Summary.

1. About two and a half or three weeks after the attachment of *Lernæopoda edwardsii* to the brook-trout, the parasite is mature for fertilization.

2. The mature male is about one third as long as the mature female. The reproductive organs of the male are paired, and are located in the posterior region of the body, between the intestine and the body wall. They consist of a testis, a coiled vas deferens, and a spermatophore.

3. The female reproductive apparatus is also paired in character and is located within the abdomen, between the intestine and the body wall. The ovaries give rise to slender oviducts, which open at the lower extremity of the abdomen. Slightly

above their terminations, the oviducts combine to form the spermatheca, which opens to the exterior through the two genital pores. Within each oviduct a spiral gland can be seen, which later develops into the cement gland.

4. In order to effect fertilization, the male makes circling movements while still attached to the host, thereby meeting a female. Then the male releases his hold on the host, attaches himself to the female, and moves down posteriorly in the vicinity of the genital pores.

5. The male bends his abdomen upward towards the female, and extrudes the spermatophores from the reproductive organs. These he manipulates with his second maxillæ and soon attaches them about the genital pores of the female.

6. The spermatozoa wander from the spermatophores into the spermatheca of the female and are stored until the eggs are ripe enough to undergo fertilization.

7. The eggs pass down the oviducts when mature, and are fertilized by the stored spermatozoa as they pass the spermatheca. The embryos pass into the external egg-sacs, where they develop into larvæ.

BIBLIOGRAPHY.

Fasten, N.

'12 The Brook Trout Disease at Wild Rose and Other Hatcheries. (The Brook Trout Disease in Wisconsin Waters.) Rep't. Wis. Fish Com., 1911–1912, pp. 12–22.

'13 The Behavior of a Parasitic Copepod, *Lernæopoda edwardsii* Olsson. Jour. of An. Beh., Vol. 3, pp. 36–40.

Gruber, A.

'79 Beiträge zur Kenntniss der Geschlechtsorgane der freilebenden Copepoden. Zeitschr. wiss. Zool., Bd. 32, pp. 407–442.

Ishizawa, C.

'91 Studies on the Reproductive Elements. I. Spematogenesis, Ovagenesis, and Fertilization in *Diaptomus sp.* Jour. of Col. of Sc., Imp. Univ., Japan, Vol. 5, Pt. I., pp. 1–34.

Miculicich, M.

'05 Zur Kenntniss der Gattung Brachiella Cuv. und der Orginisation der Lernæopodiden. Zool. Anz., Bd. 28, pp. 599–620.

Wilson, C. B.

'11 North American Parasitic Copepods. Part 9. The Lernæopodidæ. Proc. U. S. Nat. Mus., Vol. 39, pp. 189–226.

Wright, R. R.

'82 Notes on Parasitic Copepods, No. I. Proc. of Canad. Inst., Vol. I., pp. 243–254.

EXPLANATION OF PLATES.

All drawings were made with the aid of the camera-lucida. The magnification is given after the description of each figure.

Abbreviations.

a.f. = attachment filament.
ant. 1 = first antennæ.
ant. 2 = second antennæ.
b. = brain.
c. = cement-like substance of spermatophores.
can. = canal of v. d. 3.
c. g. = cement gland.
ch. = chitinous covering of spermatophore.
d. g. = digestive gland.
e. p. = ejaculatory pore.
e. s. = egg-sacs.
f. g. = frontal gland.
g. = gill.
g. p. = genital pores.
i. = intestine.
m. = mouth.
mx. 2 = second maxillæ.
mx. g. = maxillary gland.
mxp. = maxillipeds.
mxp. g. = maxillipedal gland.
o. = eggs.
od. = oviduct.
ov. = ovary.
s. = spermatheca.
s. g. = shell gland.
sp. = spermatophores.
sz. = spermatozoa.
t. = testis.
v. d. = vas deferens.
v. d. 1 = first branch of vas deferens.
v. d. 2 = second branch of vas deferens.
v. d. 3 = third branch of vas deferens.

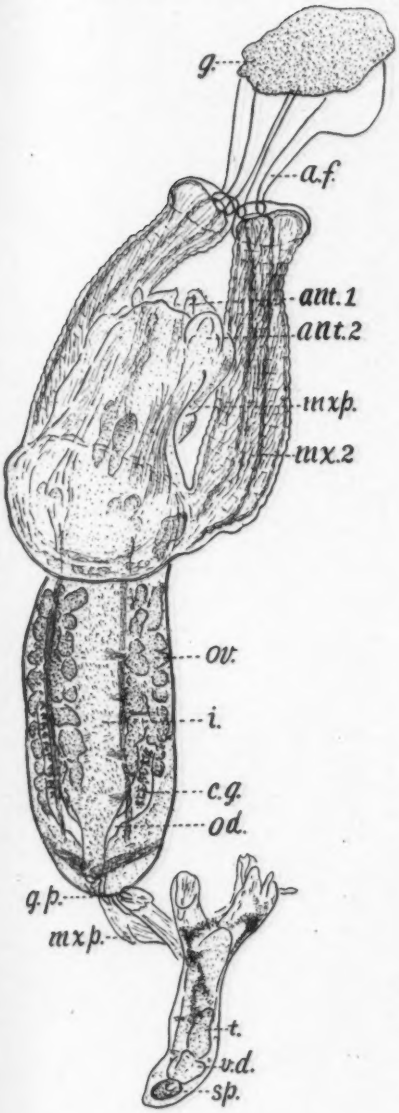
PLATE I.

Explanation of Figures.

1. Male and female copepods in position for copulation. The female is attached to the gill (*g.*) by the funnel-like attachment filament (*a. f.*). The male is attached to the female near the genital pores (*g. p.*). × 57.

2. Side view of a mature male showing the position of the reproductive organs (*t.*, *v. d.*, and *sp.*). × 120.

3. Enlarged view of the reproductive organs of the male shown in Fig. 2. × 218.



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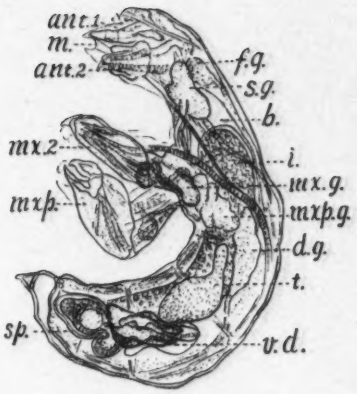

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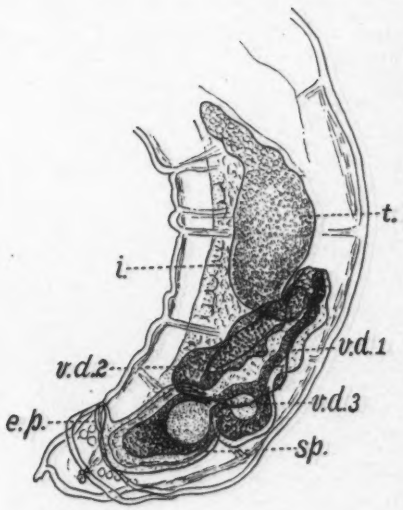

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NATHAN FASTEN.

PLATE II.

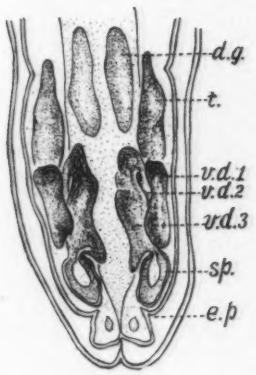
Explanation of Figures.

4. Dorsal view of the posterior extremity of a mature male, showing the position of the various parts of the reproductive organs. × 144.

5. Sagittal section through the third lobe of the vas deferens and the spermatophore. The canal-like tube (*can.*), that conducts the spermatozoa is seen in *v.d. 3*. × 560.

6. Frontal-section through the spermatophores, showing their structure. × 800.

7. Cross-section through a spermatophore slightly above its central region, showing its structure. × 1,240.



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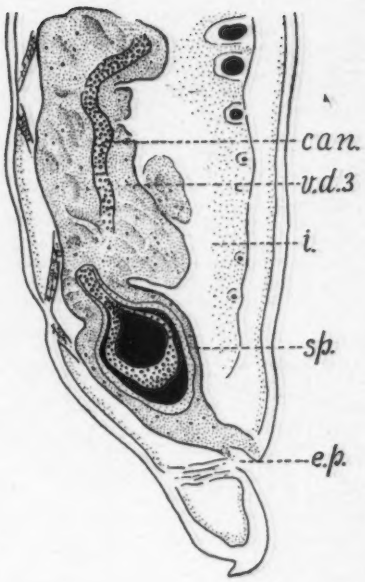

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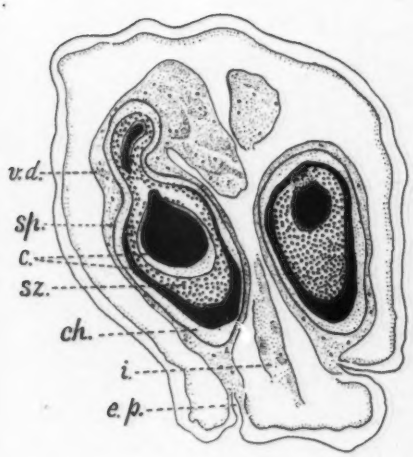

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PLATE III.

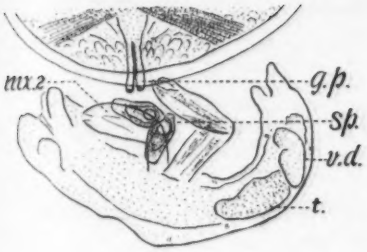
Explanation of Figures.

8. The process of fertilization. The male is here seen manipulating the extruded spermatophores (*sp.*) between his second maxillæ (*mx. 2*), in the vicinity of the genital pores (*g. p.*) of the female's body. × 100.

9. Side view of a young female after fertilization. The spermatophores (*sp.*), are attached near the genital pores. × 76.

10. Ventral view of the abdomen of an adult female showing the relation of the reproductive organs to each other. × 57.

11. Cross-section through the abdomen of an adult female in the region of the spermatheca. × 76.



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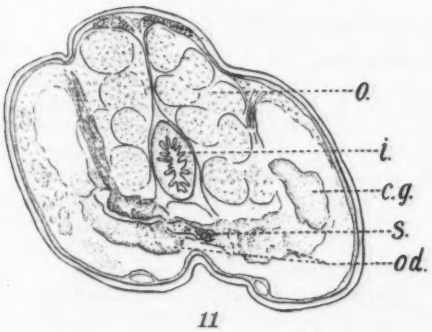

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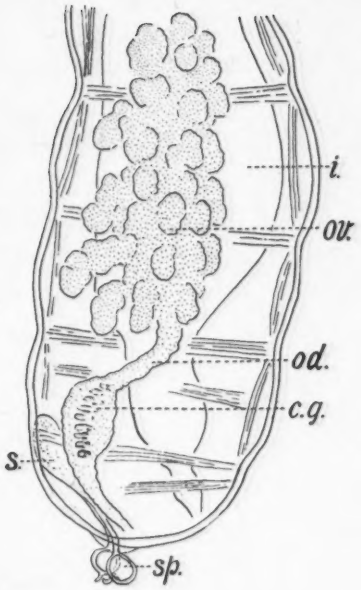

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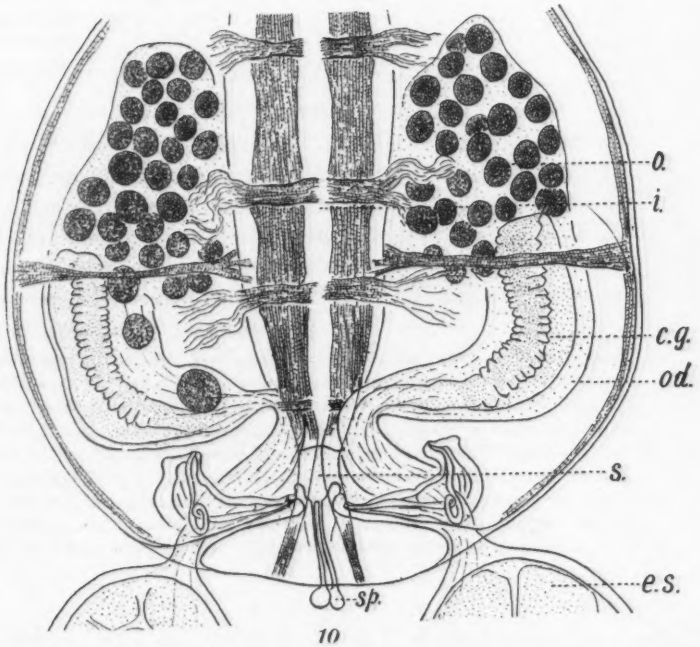

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